REMARKS

Reconsideration and withdrawal of the rejections of the application are respectfully requested in view of the amendments and remarks herein. Applicants thank the Examiner for the courtesies extended in the Office Action.

I. STATUS OF THE CLAIMS AND FORMAL MATTERS

Claim 14 has been added and is now pending. Claims 1-13 have been cancelled, without prejudice, without admission, without surrender of subject matter, and without any intention of creating any estoppel as to equivalents.

No new matter has been added.

It is submitted that the claims, herewith and as originally presented, are patentably distinct over the prior art cited by the Examiner, and the originally-filed claims and the claims herewith are and were in full compliance with the requirements of 35 U.S.C. §112. The claim presented herein are not presented for purposes of patentability within the meaning of 35 U.S.C. §§§§ 101, 102, 103 or 112. Rather, the new claim is presented simply for clarification and to round out the scope of protection to which Applicants are entitled. Furthermore, it is explicitly stated that the herewith amendments should not give rise to any estoppel.

Applicants note that the Office Action requested that the substance of the Preliminary Amendment filed February 22, 2002 be included in our response to the Office Action as the Preliminary Amendment was not signed when filed. For clarity, Applicants have incorporated the Preliminary Amendment herein, although Applicants also note that a signed version of the Preliminary Amendment was refilled on June 5, 2003, such that the Preliminary Amendment should have been properly entered prior to the issuance of the Office Action.

Accordingly, Applicants respectfully believe that no new matter is added by these Amendments, including the incorporation by reference added to the first page of the specification, because these amendments were originally presented with the application at the time of filing.

II. THE OBJECTIONS TO THE SPECIFICATION ARE OVERCOME

The Office Action objected to the specification as failing to provide proper literal antecedent basis for the claimed subject matter. The objection is respectfully traversed.

The Amendment herein adds the verbatim language of claim 14 into the specification at page 5, thereby rendering this rejection moot. It is respectfully submitted that this Amendment does not incorporate new matter into the application as the text of claim 14 was presented in the Preliminary Amendment that accompanied this application at the time of filing. Accordingly, reconsideration and withdrawal of the objection to the specification is respectfully requested.

III. THE ART REJECTIONS ARE OVERCOME

Claim 14 was rejected under 35 U.S.C. §102(b) as allegedly being anticipated by Lakshmi *et al.* Claim 14 was also rejected under 35 U.S.C. §102(b) as allegedly being anticipated by Gomez-Sanchez *et al.* The rejections are respectfully traversed, and will be addressed in turn.

In reading Lakshmi *et al.*, it is clear that this document describes testing a compound which promotes, rather than inhibits, the reductase activity of 11β-HSD1. The Lakshmi *et al.* reference concerns an *in vitro* test that is performed on a brain homogenate. As discussed on page 1, line 33 to page 2, line 5 of the specification, *in vitro* the 11β-HSD1 enzyme is bi-directional, and the enzymatic reaction can thus proceed in either direction (dehydrogenase or reductase), driven by the availability of the co-substrates (NDAP or NADPH) in the reaction. The ability of the enzymatic reaction to be driven in either reaction by the presensce of NDAP or NADPH is in fact shown in Lakshmi *et al.*, specifically in the first two paragraphs of the "Results" section on page 1743.

In contrast to Lakshmi *et al.*, the present invention is based on the discovery that while 11β-HSD1 has bi-directional activity *in vitro*, the enzyme functions as a reductase *in vivo*. Enclosed is a copy of a Declaration filed in the parent application, U.S.S.N. 09/029,535, now U.S. Patent No. 6,368,816, which describes, at paragraph 14, the difference in enzyme activity between intact cells and homogenates. Accordingly, it is clear that Lakshmi *et al.* is directed to an assay that focuses on the *in vitro* activity of an enzyme (using homogenates), and not the *in vivo* activity described by the present claim. Accordingly, reconsideration and withdrawal of the rejection is respectfully requested.

Turning now to Gomez-Sanchez *et al.*, this document describes the administration of carbenoxolone directly to the brain, which results in an increase in hypertension. This is an observation of the effect of carbenoxolone on the activity now attributed to 11β-HSD2, which

was cloned in 1993-1994, just after the publication of Gomez-Sanchez *et al.* This finding presupposes a dehydrogenase reaction, not a reductase reaction.

Furthermore, the enclosed Declaration further details the activity of 11β-HSD2, specifically in paragraph 10, which describes the identification of 11β-HSD2, and in paragraph 11, where the analogy between the dehydrogenase activity of 11β-HSD2 which protects mineralocorticoid receptors from glucocorticoids is pointed out. This protection is exactly the activity referred to in the abstract of Gomez-Sanchez *et al.*, which states "11β-HSD inactivates cortisol and corticosteroine, allowing the more abundantly produced glucocorticoids access to the mineralocorticoid receptor ... where they act as mineralocorticoids."

As evidenced by paragraphs 10 and 11 of the declaration referenced above, and by paragraph 27, which describes the link between 11β-HSD2 and hypertension, the Gomez-Sanchez *et al.* reference clearly describes 11β-HSD2, not 11β-HSD1. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. §102(b) is respectfully requested.

CONCLUSION AND REQUEST FOR INTERVIEW

In view of the amendments and remarks herewith, which are fully responsive to the rejections, the application is in condition for allowance. Consideration and entry of this paper, favorable reconsideration of the application and reconsideration and withdrawal of the objections to and rejections of the application, and prompt issuance of a Notice of Allowance are earnestly solicited.

If any issue remains as an impediment to allowance, an interview with the Examiner and the Examiner's SPE, is respectfully requested; and, the Examiner is additionally requested to contact the undersigned to arrange a mutually convenient time and manner for such an interview.

Respectfully submitted,
FROMMER LAWRENCE & HAUG LLP

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	:	Walker et al.
Serial No.	:	09/029,535
Filed	:	February 27, 1998
For	:	REGULATION OF INTRACELLULAR GLUCOCORTICOID CONCENTRATION
Examiner	:	B. Badio
Group Art Unit	:	1616

745 Fifth Avenue, New York, NY 10151

Assistant Commissioner for Patents
Washington, D.C. 2023
Dear Sir:

DECLARATION OF BRIAN R. WALKER, JONATHAN R. SECKL AND CHRISTOPHER R.W. EDWARDS

WE, BRIAN R. WALKER, JONATHAN R. SECKL, AND CHRISTOPHER R.W. EDWARDS, declare and state that:

1. We are the named inventors on the above-captioned application ("the present application") and are familiar with it and its prosecution, including the claims, and the September 1, 2000 Office Action, and the concurrently-filed Amendment, wherein the independent claims now read:

> --32. A method for inhibiting reductase activity of 11-Beta-hydroxysteroid dehydrogenase 1 (11-Beta HSD1) in an animal in need thereof in adipose or neuronal tissue of the animal comprising administering to the animal an inhibitor of said reductase activity of 11-Beta HSD1 in an amount effective to so inhibit the reductase activity of 11-Beta HSD1.
>
> 33. A method for reducing intracellular glucocorticoid concentration in an animal in need thereof in neuronal or adipose tissue comprising inhibiting the reductase activity of 11-Beta-hydroxysteroid dehydrogenase 1 (11-Beta HSD1) in said tissue.
>
> 34. A method for determining whether a compound or composition is a regulator of intracellular glucocorticoid activity in

adipose or neuronal tissue comprising determining whether said compound or composition inhibits reductase activity of 11-Beta-hydroxysteroid dehydrogenase 1 (11-Beta HSD1) in said tissue.--

2. More in particular, we are advised and therefore believe that in the September 1, 2000 Office Action, claims are rejected under 35 U.S.C. §112 because the Examiner questions "compound(s) useful as an inhibitor of" 11-Beta HSD1, enablement for such inhibitors beyond carbenoxolone, as well as to how the skilled artisan would use such an inhibitor in treating certain disorders not caused by the overproduction of cortisol; and, asserts that the specification does not enable one skilled in the art to use such inhibitors to treat certain disorders. We are also advised and therefore believe that the Office Action also rejects claims under Section 112, second paragraph, as lacking definiteness due to the recitation of "inhibitors of 11β-reductase". Moreover, we are advised and therefore believe that the Office Action rejects claims under 35 U.S.C. §102(b) as anticipated by Walker or Stewart, and under 35 U.S.C. §103 as obvious over Walker in view of Goodman and Gilman.

3. We are also familiar with the present application and the concurrently-filed Amendment, including the new claims therein, such as those set forth above, and that the arguments in that Amendment are based on our assertions herein.

4. Furthermore we, Professor Jonathan R Seckl, Dr Brian R Walker, and Professor Christopher RW Edwards, respectfully submit that we are experts in the field to which the present application pertains and particularly in the field of 11β-hydroxysteroid dehydrogenases. In addition to brief *curricula vitae* that may be attached for any one or all of us, we Professor Jonathan R Seckl and Dr Brian R Walker, have both been active researchers in this field for more than 10 years and, together and separately, have published more than 200 relevant primary articles in peer-reviewed journals and more than 80 review articles and contributions to books. We have obtained very substantial external research funding for our work in this area in open competition. We both lead research groups within the University of Edinburgh in which we supervise more than 40 full-time research staff who are investigating aspects of glucocorticoid biology, including 11β-hydroxysteroid dehydrogenases. We are both asked regularly to speak to the subject of 11β-hydroxysteroid dehydrogenase biology at national and international scientific meetings. Indeed, I, Professor Jonathan R. Seckl, am the Moncrieff-Arnott Professor of Molecular Medicine (and Professor of Endocrinology) at the University of Edinburgh, Molecular

Medicine Centre, Western General Hospital, where I am Head of the Molecular Medicine Center (~150 scientists) and Head of the Molecular Endocrinology Laboratory (~40 scientists). I have more than 20 years experience in medicine and endocrinology research, have published more than 150 peer-reviewed primary research papers, as well as over 50 learned reviews and book chapters within the field of endocrinology, the vast majority address glucocorticoid biology and metabolism. I, Dr. Brian R. Walker, invite review of my attached brief *curriculum vitae*, and state that am a Reader and British Heart Foundation Senior Research Fellow in the Department of Medical Sciences, Western General Hospital, University of Edinburgh. And, I, Professor Christopher R.W. Edwards, am Vice-Chancellor of the University of Newcastle upon Tyne, past Principal of the Imperial College School of Medicine, London, and past Dean of the Faculty of Medicine in the University of Edinburgh and Professor of Clinical Medicine in that same University, with over 30 years experience in Medicine and Endocrinology. I have published on many aspects of endocrinology (over 400 publications including articles in Nature, JCI, Lancet and NEJM), especially mineralocorticoid hypertension and mechanisms conferring specificity on the mineralocorticoid receptor.

5. Accordingly, in view of our education, training and experience, we are considered by our peers to be experts in the field to which the present application pertains, and qualified to knowledgeably characterize the art to which the invention in the present application relates, and to speak as to the present application, and the invention claimed, including being qualified to present expert opinions about the present invention and literature in support of it, and documents cited against the present invention. Moreover, we respectfully submit that we are qualified to state the knowledge in the art, and that which the skilled artisan would not have required any undue experimentation to practice, e.g., the enablement and the written description in the present application, and what the skilled artisan would have been taught, as well as what would have been obvious and nonobvious to the skilled artisan.

6. Thus, this Declaration is intended to assert the sufficiency of the enablement, written description, novelty and inventive step (nonobviousness) of the claimed subject matter of the present application (as of original filing of the patent specification in August 1995), i.e., to respond to the rejections of the present application under 35 U.S.C. §§112, first and second paragraphs, and 102 and 103; which rejections we respectfully request be reconsidered and withdrawn in view of this Declaration and the attachments hereto. To this end we review the

state of knowledge in the art in 1995, demonstrate the inventive aspects in our patent specification, and further illustrate these with reference to more recent data. All documents cited herein are listed on a reference list that appears before the closing paragraph and our signatures. All documents cited herein are incorporated herein by reference, and a copy of those documents indicated in the following text as attached is included with this Declaration, to assist the Examiner in confirming our assertions and discussions herein.[a] The Examiner is respectfully requested to consider and make of record documents cited herein.

BACKGROUND

7. Glucocorticoids are natural and synthetic steroid hormones. Physiologically they regulate basal metabolism in many tissues and, critically, underpin the organism's response to stressful stimuli. Thus, a rise in plasma glucocorticoid levels increases the availability of metabolic fuels, raising glucose and lipid levels in the blood, and elevates blood pressure. Glucocorticoids also inhibit processes not essential to immediate survival, such as inflammation and immune responses, growth, reproductive, and digestive functions. Pathological chronic glucocorticoid excess (Cushing's syndrome) presents with hypertension, type 2 diabetes, insulin resistance, central obesity, depression and memory decline. More subtle forms of glucocorticoid excess have been proposed to underpin the much more frequent disorders of hypertension, type 2 diabetes/insulin resistance, dyslipidaemias, obesity and mood disorders. Therapeutically, general systemic manipulation of glucocorticoid levels (up or down) has widespread adverse effects that usually counterbalance the benefits.

8. Glucocorticoids and mineralocorticoids, like other steroids, are lipophilic and readily access their intracellular receptors. Until a decade or so ago, it was thought that the main determinants of corticosteroid action were the levels of hormones in the blood, their binding by plasma proteins (eg corticosteroid binding globulin) and the varying densities of receptors in target tissues. However, it has become apparent that an additional and important level of control is exerted by pre-receptor metabolism of ligands by tissue-specific enzymes. Such modulation of steroid action by local metabolism has been described for other hormones, including androgens (5α-reductases), oestrogens (17β-hydroxysteroid dehydrogenases and aromatase), and thyroid hormones (5'-monodeiodinases). For glucocorticoids, the key enzymes are 11β-hydroxysteroid

[a] With about 70 documents cited herein, it was believed to be possibly overwhelming to have attached them all; but we gladly would have done so. Accordingly, in addition to the documents indicated as attached, we will gladly

dehydrogenases (11β-HSDs). Understanding the tissue-specific functions of 11β-HSDs – including through the teachings in the present application - has led to new insights into pathophysiology of common diseases. As will be clear from the discussion herein, we have played a major role in this area of reasearch.

HISTORY

9. Almost 50 years ago Amelung and colleagues [1], discovered the enzymic interconversion of active 11-hydroxy glucocorticoids (cortisol, corticosterone) and inert 11-keto forms (cortisone, 11-dehydrocorticosterone). In the mid-1980s Monder and co-workers in New York purified an NADP(H)-dependent 11β-HSD activity from rat liver which catalysed both 11β -dehydrogenation of cortisol to inert cortisone and also the 11β-reduction of cortisone to active cortisol [2]. At this stage, it was suggested that the 11β-dehydrogenase and 11β-reductase activities of this enzyme 'complex' reside in different proteins [3]. 11β-HSD was thought to represent one of several arcane pathways for clearance of glucocorticoids and no more specific function was ascribed to it. However, in the late 1980s Edwards and colleagues in Edinburgh and Funder et al in Melbourne recognised the key physiological importance of the inactivation of cortisol to cortisone. These workers discovered that 11β-HSD activity in the distal nephron could explain the 'mineralocorticoid receptor paradox' [4;5]. This arose from findings that purified or recombinant mineralocorticoid receptors were non-selective *in vitro* and bound the glucocorticoids cortisol and corticosterone with equal affinity to the physiological mineralocorticoid aldosterone [6]. Nevertheless, the same receptors *in vivo* were aldosterone-specific in the face of many fold molar excess of glucocorticoid [7]. The explanation lay in 11β-HSD which rapidly inactivated glucocorticoids in aldosterone target cells in the distal nephron, thus allowing selective access of aldosterone to mineralocorticoid receptors. In the congenital absence of this activity (the syndrome of 'apparent mineralocorticoid excess')[8], or with liquorice-based inhibitors of 11β-HSD [9], glucocorticoids illicitly occupy mineralocorticoid receptors causing sodium retention, hypokalemia, and hypertension.

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provide a copy of any other document cited herein upon request.

DISCUSSION – THE PATENTABILITY OF THE PRESENT INVENTION

Two isozymes of 11β-HSD

10. Monder and colleagues then cloned a cDNA from a λGT11 expression library using antibodies raised against their 11β-HSD purified from rat liver [10]. This cDNA hybridised with a product highly expressed in rat kidney [11;12] and was thought to represent the active 11β-dehydrogenase. However, it became clear that this enzyme could not explain mineralocorticoid receptor 'protection' in the distal nephron. For example, it was expressed widely (including in hippocampus, where mineralocorticoid receptors are not selective for aldosterone), was of low affinity (micromolar Km for active 11-hydroxysteroids), and did not match the regulation or co-factor preference of the 11β-dehydrogenase activity in distal nephron. These discrepancies were resolved in 1993/94 with the purification [13] and cloning [14;15] of a second isozyme, 11β-HSD2. 11β-HSD2 is highly expressed only in classical aldosterone-selective target tissues (distal nephron, colon, sweat glands) and the placenta. 11β-HSD2 cDNA encodes a high affinity, NAD-dependent dehydrogenase which rapidly inactivates glucocorticoids with a low nanomolar K_m. This enzyme has negligible 11β-reductase activity. Mutations in the 11β-HSD2 gene are seen in patients with the congenital syndrome of apparent mineralocorticoid excess [16]. Mice homozygous for targeted disruption of the 11β-HSD2 gene [17] recapitulate the features of glucocorticoid-dependent mineralocorticoid excess. It is therefore quite clear that 11β-HSD2 is the enzyme responsible for protecting mineralocorticoid receptors from glucocorticoids *in vivo*.

Monder's rat liver enzyme, 11β-HSD Type 1: characterising the functions of 11β-HSD1

11. By this time, in the mid-1990s, extensive studies had been performed using cDNA and antibodies raised against 11β-HSD1, initially in the mistaken belief that this gene product encoded the 11β-dehydrogenase which protects renal mineralocorticoid receptors from glucocorticoids. 11β-HSD1 was found to be widely expressed, most notably in liver, lung, adipose tissue, vasculature, ovary and the CNS. High expression was also observed in the kidney and testis in the rat, but not in the mouse. In many of these sites there is negligible expression of mineralocorticoid receptors, but glucocorticoids play a key role in regulation of metabolism through activation of relatively low affinity glucocorticoid receptors. By analogy with the role of 11β-HSD2 in protecting renal mineralocorticoid receptors from glucocorticoids, 11β-HSD1 was proposed by several investigators to be a predominant 11β-dehydrogenase protecting

glucocorticoid and/or mineralocorticoid receptors from glucocorticoids in other tissues. Examples include the blood vessel wall [18-20], heart [21], breast [22], adipose tissue [22;23], testicular Leydig cell [24;25], ovary [26], and brain [27;28]. This interpretation of a predominant 11β-dehydrogenase activity of 11β-HSD1 in many tissues is reflected in contemporary reviews of the mid-1990s, eg [23].

12. For instance, Monder and White in "11β-Hydroxysteroid dehydrogenase," **Vitamins and Hormones** 1993; **47**: 187-271 (copy attached), outright stated at page 191 that "11-HSD in human adipose tissue has been reported to catalyze only oxidation." Moreover, to this day, predominant 11β-dehydrogenase activity of 11β-HSD1 is invoked by investigators interpreting data in testicular Leydig cells [29-32], bone cells [33], and brain [34]. And, note further that Monder and White, *supra* at page 189 teach that the dominant activity of 11β-HSD1 in most tissues other than the liver is 11β-hydroxy oxidation.

13. There was, however, literature which suggested that 11β-HSD1 could function as an 11β-reductase under some circumstances. Older literature, and literature from studies in the early 1990s, discussed that 11β-HSD1 in the liver does not always predominantly function as a 11β-dehydrogenase; specifically, in the liver it was known to have considerable 11β-reductase activity. Experiments in isolated perfused cat [35] liver suggested that 11β-HSD1, which is the only isozyme expressed in the liver, is indeed a predominant 11β-reductase with a high capacity for reactivating 11-ketosteroid substrate over a broad range of substrate concentrations. More recent work confirms this in rat liver [36]. These findings can be extrapolated to human liver *in vivo*, since historical work suggests that, on oral administration, cortisone (the first pharmacological glucocorticoid used in man) is rapidly activated to cortisol. Indeed, recent studies confirm that very little oral cortisone reaches the systemic circulation [37] and it was known that hepatic vein cortisol/cortisone ratios are very high [38]. Predominant 11β-reductase activity was also found in primary cultures of cells from liver [39]. What was not known in 1995 was whether this predominant 11β-reductase activity of 11β-HSD1 is present in many other tissues besides the liver. Indeed, the prevailing view, outlined above, was that the enzyme was predominantly an 11β-dehydrogenase in tissues other than the liver.

11β-Dehydrogenase or 11β-Reductase in non-hepatic tissues?

14. In original purification studies, the 11β-HSD1 in the liver was shown to be bi-directional although, in contrast with its 11β-dehydrogenase activity, the 11β-reductase activity was unstable *in vitro* [2]. As a result of this lack of stability of 11β-reductase activity, many investigators deduced from the predominant 11β-dehydrogenase activity in semi-purified enzyme preparations from other tissues that the enzyme was also a predominant 11β-dehydrogenase *in vivo*. Since 1995 (after the effective filing date of the present application), a series of studies have suggested that the enzyme prefers the reductase direction in many tissues unless cells are disrupted. This applies in primary cultures of cells not only from liver [39], but also from adipose tissue [40], lung [41], CNS [42], vascular smooth muscle [43], and Leydig cells [44] (although in Leydig cells and blood vessels directionality of the enzyme remains controversial [32;45]). This striking change in directionality between intact cells and homogenates has never been satisfactorily explained, but may reflect the specific intracellular localisation of 11β-HSD1 in the inner leaflet of the endoplasmic reticulum, where neighbouring enzymes may be powerful generators of the reduced co-substrate NADPH. Short-term post-translational changes such as enzyme phosphorylation may also be pertinent, particularly to the apparent instability of the 11β-reductase activity in homogenates, but remain to be investigated. Alternative explanations, such as longer-term post-translational modifications (varying N-linked glycosylation)[46] would not explain why 11β-HSD1 activity is overwhelmingly reductive in intact cells and then shows predominant dehydrogenation in homogenates of these same cells. Importantly, because no mechanism is known which explains the directionality of activity of 11β-HSD1 activity *in vivo*, and because reports of predominant 11β-dehydrogenase activity continue to be published in some tissues (eg Leydig cell [32]), it cannot be assumed that 11β-HSD1 is a predominant reductase in all cells; each tissue and cell must be examined in turn.

15. Considering specifically again the Office Action and the present invention, in two tissues in particular, namely, adipose tissue and neuronal tissue, the predominantly 11β-reductase activity of 11β-HSD1 was disclosed for the first time in the British application upon which the present application claims priority; namely, our original patent specification of August 1995. This was wholly unexpected in 1995; and again, the enzymatic activity of 11β-HSD1 in a particular tissue, such as adipose or neuronal tissue, could not be predicted from the activity of

this enzyme in another tissue, such as liver tissue; and, that the art had indicated that the activity of this enzyme in adipose and neuronal tissue was a dehydrogenase activity, contrary to the present invention which involves the finding that in adipose and neuronal tissue 11β-HSD1 has predominantly reductase activity. Interestingly, on information and belief, two (2) years after the effective filing date of the present application, Stewart and colleagues identified the predominantly 11β-reductase activity in adipose tissue in 1997, and, attempted to patent this finding; but, their British application on our prior discovery was not pursued, after our prior disclosure was identified to them. Further, Katz et al., writing in 1999 – nearly four years after the effective filing date of the present application – presented data which they argued was novel in that it "demonstrated 11 beta HSD oxo-reductase activity in subcutaneous abdominal adipose tissue, which may be increased in obesity," further showing the novelty and nonobviousness of our invention (*see* Katz et al., "An in vivo study of the cortisol-cortisone shuttle in subcutaneous abdominal adipose tissue," Clin Endocrinol (Oxf) 50(1): 63-8 (1999), copy of Abstract attached).

16. Accordingly, considering the office action, it is duly noted that the teaching in the art has been contrary to the present invention. More in particular, the present invention involves the discovery that 11β-HSD1 acts as a predominant 11β-reductase in adipose and neuronal tissues, rather than being either bi-directional or predominantly an 11β-dehydrogenase - contrary to the literature that teaches that 11β-HSD1 acts as a dehydrogenase or is bi-directional in these tissues. Simply, in 1995, at the time of our effective filing date, it was widely accepted that 11β-HSD1 was a reductase in the liver and a dehydrogenase everywhere else. *WE THEREFORE RESPECTFULLY CONCUR WITH THE EXAMINER THAT* "the effects of modulation of the activity of an enzyme, including 11β-reductase, in a given tissue cannot be predicted based on its effects in another tissue"; *and, as demonstrated herein, we respectfully disagree with the Examiner that* "the effect of a compound on an enzyme activity would be expected to be the same no matter which tissue said enzyme is found in." For instance, from the discussion so far, it is respectfully submitted that it is clear that an inhibitor of 11 β-HSD1, such as carbenoxolone, in certain tissues will inhibit dehydrogenase activity resulting in elevated cortisol concentrations, and in other tissue it will inhibit reductase activity resulting in lower cortisol concentrations. Simply, we have shown that the enzymatic activity of 11β-HSD1 in a particular tissue, such as adipose or neuronal tissue, could not be predicted from the activity of this enzyme in another tissue, such as liver tissue; and, that the art had indicated that the activity of this enzyme in

adipose and neuronal tissue was a dehydrogenase activity, contrary to the present invention which involves the finding that in adipose and neuronal tissue 11β-HSD1 has predominantly reductase activity. Therefore, while carbenoxolone or other inhibitors of 11β-HSD1[b] would be expected to inhibit 11β-HSD1, one could not predict the effect of the inhibitor on enzymatic activity, e.g., whether reductase activity or dehydrogenase activity would be inhibited, since the enzymatic activity of 11β-HSD1 in any given tissue could not have been predicted.

Further evidence for discrepancies in 11β-HSD1 function between tissues: tissue-specific regulation

17. Studies of regulation of 11β-HSD1 expression and activity in different tissues reinforce the concept that the function of the enzyme cannot be extrapolated from one tissue to another. From extensive studies of regulation, it is clear that this is both species- and tissue-specific. For example, estradiol is a potent down-regulator of 11β-HSD1 expression in rat liver[47] but not rat hippocampus or human liver [48]. In comparisons of human hepatocytes [49] and adipose cells [50] in primary culture, Stewart's group have reported that the enzyme in adipose cells is highly regulated (eg by IGF1 and tumour necrosis factor α) but the enzyme in hepatocytes is unaffected by these manipulations. Such tissue-specificity is probably determined by tissue-specific responses to these regulators, including differences in direct regulation of 5' promoter activity in the 11β-HSD1 gene for example by the C/EBP family of transcription factors [51].

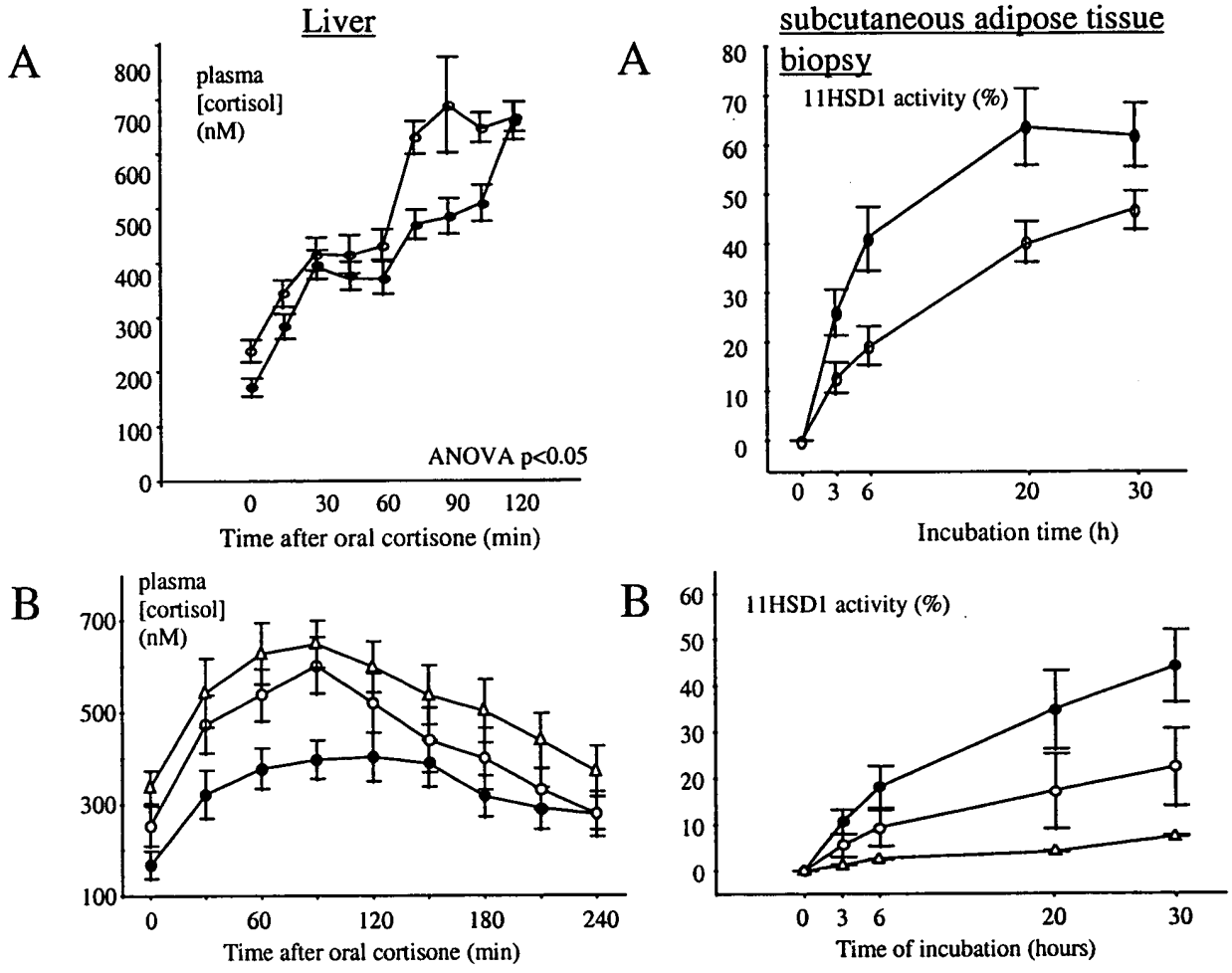
18. Further insights into tissue-specific dysregulation have emerged from very recent studies of 11β-HSD1 activity in animals and patients with insulin resistance syndromes in which we propose that inhibition of 11β-HSD1 would be beneficial. Studies in leptin-resistant obese Zucker rats [52], in obese humans [53;54], and in patients with type 2 diabetes mellitus [55] show that hepatic 11β-HSD1 activity is down-regulated. However, in the same animals [52] and patients [54] (Rask et al. "Tissue-specific dysregulation of cortisol metabolism in human obesity," manuscript attached) (unpublished data in type 2 diabetes mellitus shown in Figure 1), 11β-HSD1 activity in adipose tissue is enhanced rather than impaired. Thus, the predicted effects of inhibition of 11β-HSD1 cannot be extrapolated from one tissue to another, since in these syndromes of insulin resistance we would expect 11β-HSD1 inhibition to be less effective in liver and more effective

[b] We shall also herein demonstrate that inhibitors of 11β-HSD1, other than carbenoxolone, were known, and that it did not, in view of the knowledge in the art, require any undue experimentation to identify those other inhibitors and administer them.

in adipose tissue than in healthy subjects. Similarly, one could not predict the effect of an inhibitor of 11β-HSD1 on enzymatic activity, e.g., whether reductase activity or dehydrogenase activity would be inhibited, since the enzymatic activity of 11β-HSD1 in any given tissue could not have been predicted.[c]

[c] Of course, once one skilled in the art identified the enzymatic activity of 11β-HSD1 in a specific tissue, it did not require undue experimentation to determine whether a known inhibitor of this enzyme would likewise inhibit the particular enzymatic activity of the enzyme in that specific tissue.

Figure 1 Tissue-specific differences in 11β-HSD1 activity in A) type 2 diabetes mellitus and B) obesity.
Liver 11β-HSD1 activity was measured *in vivo* by conversion of cortisone administered by mouth into cortisol in peripheral plasma on first-pass metabolism through the liver. Adipose tissue 11β-HSD1 was measured by incubation of homogenised subcutaneous adipose tissue obtained by biopsy. Data are mean ± SEM.
In figures A, subjects were diabetic patients (closed circles) or healthy controls (open circles). 50 subjects had liver enzyme activity and 12 had adipose enzyme activity measurements. In figures B, subjects were from lowest (open triangle), middle (open circle), and highest (filled circle) tertiles of body mass index. 34 subjects had liver enzyme activity and 16 had adipose enzyme activity measurements.
11β-HSD1 activity is down-regulated in liver and up-regulated in adipose tissue in patients with obesity and in patients with type 2 diabetes mellitus (the latter were selected to be 'lean', so that this difference is not explained by obesity in the diabetic patients). (see also attached manuscript by Rask et al.) [54].



Evidence that 11β-HSD1 amplifies glucocorticoid action in different tissues

19. From the above, it appears that inert substrate can be reactivated by the predominant 11β-reductase activity of 11β-HSD1 in many tissues *in vivo*. However, just because an enzyme is present within a tissue, this does not mean that it influences local corticosteroid receptor activation. For example, other hepatic enzymes which metabolise cortisol in liver, such

as A-ring reductases (5α- and 5β-reductases), have not been shown to modulate local glucocorticoid receptor activation. The reason for this remains unclear because we do not yet understand the intracellular 'anatomical' relationship between 11β-HSDs and the receptors they modulate. A large number of inhibitors of 11β-HSDs were identified in the art before 1995 [23] and evidence collected using these compounds. As discussed above, much of this evidence has been interpreted as supporting predominant 11β-dehydrogenase activity of 11β-HSD1 protecting corticosteroid receptors in sites such as vascular smooth muscle [18-20;56] and Leydig cell [25;32;57]. The inference that predominant 11β-reductase activity of 11β-HSD1 amplifies local glucocorticoid receptor activation was first hinted at in liver in the early 1990s, but not established for any other tissue before 1995.

Liver

20. In liver, glucocorticoids oppose the actions of insulin, for example by up-regulating expression of the rate-limiting enzyme for gluconeogenesis, phosphoenol-pyruvate carboxykinase (PEPCK). Experiments in healthy humans using the liquorice derivative carbenoxolone to inhibit 11β-HSD1 activity [58] showed enhanced insulin sensitivity, as measured in a euglycaemic hyperinsulinaemic clamp study, attributed to lower intra-hepatic cortisol levels. Subsequent studies have confirmed this. As disclosed in the patent specification, in male rats, estradiol potently down-regulates 11β-HSD1 expression [47] and, only in the presence of glucocorticoids, also down-regulates PEPCK expression [59]. Such indirect studies, as well as the use of liquorice-based inhibitors, indicate that impaired activity of 11β-HSD1 in liver is associated with features of reduced glucocorticoid action and increased insulin sensitivity in hepatocytes.

21. To explore this further, 11β-HSD1 knock-out mice have been generated [60], as disclosed for the first time in the present application. These mice appear to develop normally and are viable, fertile and normotensive. This model shows that 11β-HSD1 is the sole major 11β-reductase, at least in mice, since adrenalectomised 11ß-HSD1 knockout mice cannot convert administered inert 11-dehydrocorticosterone to active corticosterone. However, despite slightly elevated basal plasma corticosterone levels (see below), 11β-HSD1 -/- mice have a phenotype compatible with impaired intracellular glucocorticoid regeneration and reduced antagonism of insulin action. For example, they show impaired induction of PEPCK and glucose-6-phosphatase in the liver on fasting and a lesser hyperglycemic response to stress or induction of obesity [60].

Adipose tissue

22. By contrast with the existing evidence that 11ß-HSD1 was a predominant 11ß-reductase enzyme in liver in 1995 (see above), in adipose tissue the enzyme had not been studied in detail and was thought to be a predominant dehydrogenase [23]. Consistent with our disclosure of its predominant 11ß-reductase activity in the present application, we have evidence of its important role in regulating adipose lipid metabolism. Specifically, administration of a known amount (100 mg every 8 hours for 7 days) by a known route (orally) of a known inhibitor (carbenoxolone) to humans (both healthy volunteers and patients with type 2 diabetes mellitus) resulted in changes in lipid metabolism, as described in Figure 2. Further, mice with transgenic knockout of 11ß-HSD1 have similar beneficial changes in lipid profile, as described in detail in the attached manuscript by Morton et al., "11ß-hydroxysteroid dehydrogenase Type 1 Null Mice have an Atheroprotective Lipid Profile", and reduced leptin mRNA, a glucocorticoid-stimulated gene selectively expressed in adipose tissue (Figure 2). These observations are consistent with what is taught and described in our original patent specification would result from impaired regeneration of glucocorticoid by 11β-HSD1 in adipose tissue.

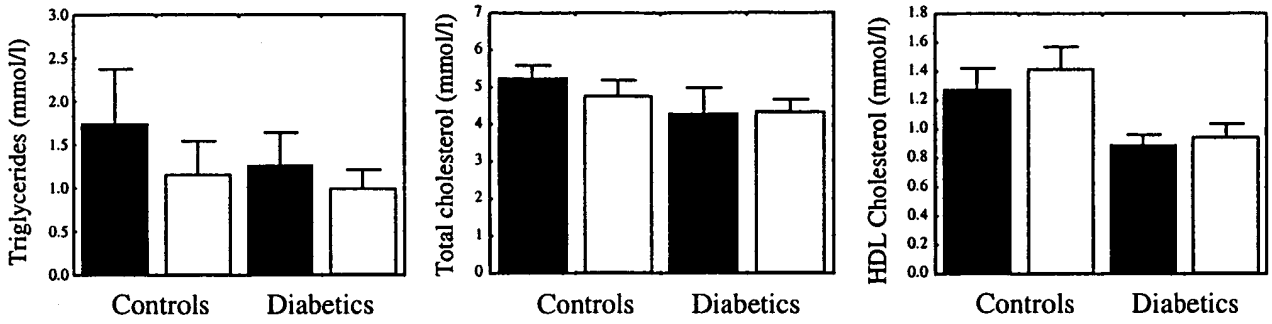
23. Moreover, in the original interpretation of the insulin sensitising effect of the 11ß-HSD inhibitor carbenoxolone in healthy humans (see our prior publication Walker et al., "Carbenoxolone Increases Hepatic Insulin Sensitivity In Man: *In vivo* Evidence That Ligand Metabolism Modulates Activation of Glucocorticoid Receptors," J. Endocrinology 104 supplement, 1994; and [58]), we proposed that this was mediated by lowered intra-hepatic glucocorticoid levels. This interpretation was based on the observation that carbenoxolone did not alter glucose uptake in the forearm circulation in man. However, forearm blood flow is almost entirely through skeletal muscle. Therefore, these published measurements did not address the possibility that enhanced sensitivity to insulin in adipose tissue might contribute to the effect of carbenoxolone. Consistent with the disclosure in the present application, our most recent, unpublished, studies (see attached manuscript by Morton et al and Figure 2) evince that an important site of action of carbenoxolone in healthy patients and in patients with insulin resistance syndromes (exemplified here by type 2 diabetes mellitus) is to inhibit 11ß-reductase activity in adipose tissue, thereby enhancing altering adipose metabolism and producing beneficial changes in lipid profile. In parallel with these changes in lipid metabolism we can expect enhanced peripheral glucose uptake in adipose tissue. Thus, the findings in Morton et al.

and in Figure 2 are consistent with the teachings in the present application concerning the utility of 11β-HSD1 inhibition in lowering intracellular cortisol concentrations in adipose tissue, i.e. the beneficial effect of the 11ß-HSD1 inhibitor, e.g., carbenoxolone, is mediated at least in part in adipose tissue. Note especially, that this beneficial effect in adipose tissue could not be extrapolated from prior art data available for liver before 1995, which specifically rejected the hypothesis that enhanced glucose uptake in peripheral tissues (skeletal muscle or adipose tissue) was an important effect of carbenoxolone. Further, one might also argue that the prior art involving the liver was inconclusive: although it may have shown that carbenoxolone could enhance insulin sensitivity (*see* Walker et al., "Carbenoxolone Increases Hepatic Insulin Sensitivity In Man: *In vivo* Evidence That Ligand Metabolism Modulates Activation of Glucocorticoid Receptors," J. Endocrinology 104 supplement, 1994), it did not demonstrate conclusively that this was mediated by changes in hepatic intracellular glucocorticoid concentration. From the above, our interpretation today is that the enhancement of insulin sensitivity by carbenoxolone administration in humans may be in large part accounted for by changes in intra-adipose glucocorticoid concentration – something consistent with the teachings in the application and something not taught or suggested in the art- and that the prior art teachings of Stewart and Walker (cited in the Office action) have turned out to be possibly incorrect[d].

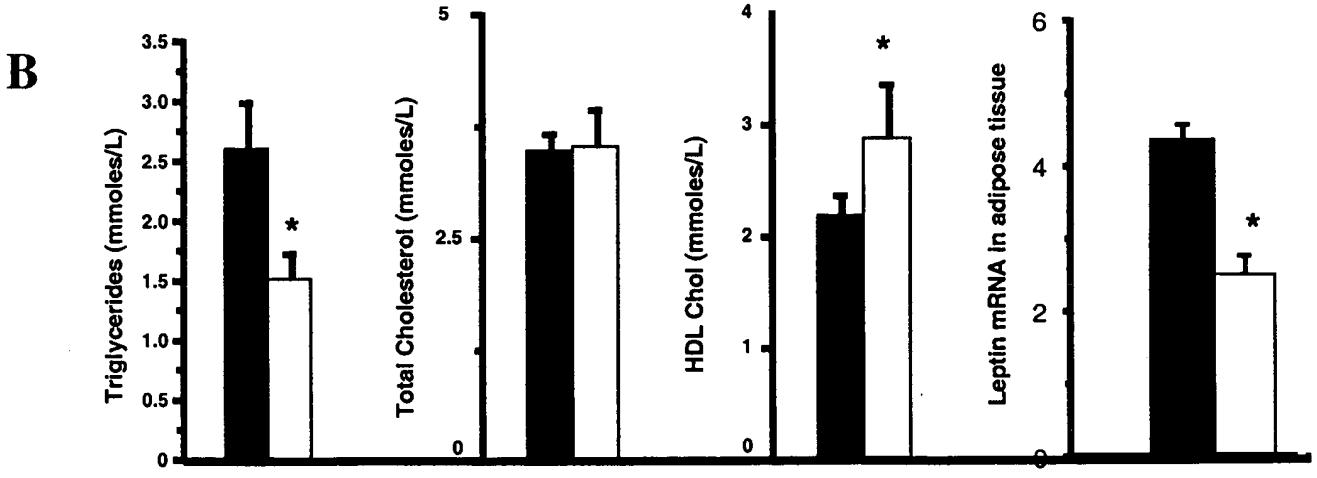
[d] The Examiner is respectfully requested to consider that as the inventive entity on the present application involves co-authors on the Stewart et al. and Walker et al. articles, and that the inventors on the present application are all academics, for us to assert that previous publications by named inventors on the present application are incorrect, it is respectfully submitted, should be considered to be highly probative because seldom does an academic admit that any one of his publications are incorrect, especially in the "publish or perish" world of academia.

Figure 2:
Influence of inhibition of 11β-HSD1 on adipose metabolism

A) Effect of administration of carbenoxolone on fasting plasma lipids in healthy humans and patients with type 2 diabetes mellitus.
6 men with type 2 diabetes mellitus and 6 healthy controls were administered placebo (filled bars) and carbenoxolone (open bars) in a randomised double-blind crossover study, as known in the art. Fasting levels of plasma lipids are shown. Carbenoxolone did not affect total cholesterol, but lowered triglyceride and raised HDL (high density lipoprotein) cholesterol concentrations. These beneficial effects are consistent with an effect to lower cortisol concentrations within adipose tissue.



B) Plasma lipids and adipose tissue leptin mRNA levels in mice with transgenic knockout of 11β-HSD1.
Plasma lipids are shown from 6 wild type (filled bars) and 6 11β-HSD1 -/- knockout animals on ad libitum diet. Data are mean ± SEM. * indicates $p<0.05$ versus wild type. 11β-HSD1 knockout animals have lower triglyceride levels, and higher HDL cholesterol levels, but not difference in total cholesterol levels. These effects are consistent with lowered corticosterone concentrations in adipose tissue, as explored in detail in attached manuscript by Morton et al.. This contention is supported by the reduced expression of leptin mRNA (a glucocorticoid-stimulated transcript) in adipose tissue in 11ß-HSD1 knockout mice.



24. Accordingly, the beneficial effects of administering an 11ß-HSD1 inhibitor for inhibition of reductase activity in adipose tissue, e.g., of 11β-HSD1 inhibition in adipose tissue, could not have been extrapolated from prior art relating to the liver; and, the teachings in the

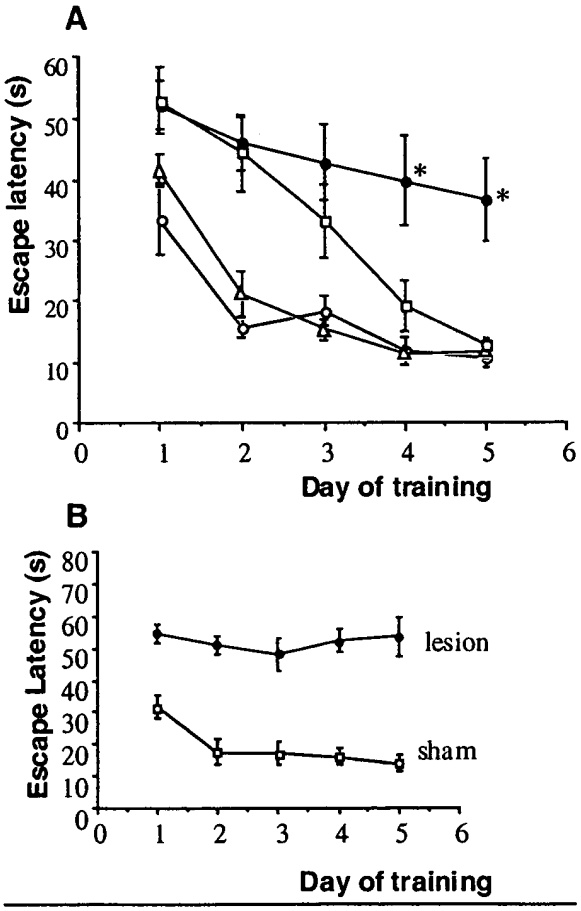
present application, for instance, the administration of an 11β-HSD1 inhibitor, e.g., carbenoxolone, for inhibition of 11β-HSD1 in adipose tissue with beneficial results, can be practiced without undue experimentation. Thus, the present invention is indeed novel and nonobvious, is described in the present application, and is enabled, e.g., can be practiced without undue experimentation.

Brain

25. In 1995, the view in the literature was that 11β-HSD1 was a predominant 11β-dehydrogenase in several brain regions which protected corticosteroid receptors from activation by glucocorticoids [23;28;61-63]. The disclosure in the present application of predominant 11β-reductase activity of 11β-HSD1 in cultured hippocampal neurons was entirely novel and has since been published [42]. In the CNS, glucocorticoids regulate key developmental, metabolic, neurotransmitter and structural functions, particularly in neurons. Chronic glucocorticoid excess has deleterious effects most notably in the hippocampus which has a very high density of corticosteroid (glucocorticoid) receptors. In hippocampal cells, 11-dehydrocorticosterone is as potent as corticosterone in potentiating excitatory amino acid neurotoxicity *in vitro*, an effect lost on inhibition of the enzyme [42]. These findings, disclosed in the 1995 application, also indicated that inhibition of this hitherto unexpected reductase activity might be of utility in treating or preventing glucocorticoid excess-associated disorders of the brain, notably age-related cognitive (learning and memory) dysfunctions and affective (depressive) illnesses. Further studies in 11β-HSD1 null mice support the notion that reduction of the enzyme attenuates the deleterious effects of chronic glucocorticoid excess upon cognitive function with aging *in vivo* (*see* attached manuscript by Yau et al., "Lack of tissue glucocorticoid reactivation in 11β-hydroxysteroid dehydrogenase type 1 knockout mice prevents age-related cognitive impairment"). The 11ß-HSD1 knock-out mice do not show the loss of the ability to learn in the watermaze (a test of hippocampus-associated cognitive function) that otherwise occurs in the majority of aged wild-type mice (*see* Fig 3 below), showing that administration of an 11ß-HSD1 inhibitor for inhibition of 11ß-HSD1 in neuronal tissue can indeed have beneficial results, as taught in the present application. Furthermore, the beneficial effects of administering an 11β-HSD1 inhibitor for inhibition of reductase activity in neuronal tissue, e.g., of 11β-HSD1 inhibition in neuronal tissue, cannot be extrapolated from observations in the liver (as argued above). Thus, the present

invention is indeed novel and nonobvious, is described in the present application, and is enabled, e.g., can be practiced without undue experimentation.

Figure 3: (A) Impaired learning of aged wild type, but not 11ß-HSD-1 knockout mice in the watermaze. Mice were trained for 5 consecutive days (4 trials/day) to find and escape onto a randomly located flagged hidden platform. Young wild type 129/Ola mice (black-open circles) and young 11ß-HSD1 knockout (blue-open triangles) learned the task well, swimming to the platform within 15 seconds after 5 days of training. Aged (18-20 month) wild type (red-closed circles) mice failed to learn the task over 5 days of training. In contrast, aged (18-20 month) 11ß-HSD1 knockout mice (green-open squares) learned the task as well as the young mice by the 4th and 5th days of the trials.* P<0.05 compared to all other groups.
(B) Failure to learn the proximally cued watermaze task following experimental hippocampal lesions in young 129/Ola mice, indicating hippocampal-dependence of this task in this strain.



11β-HSD1 in human disease; patients in need of 11β-HSD1 inhibition

26. Having demonstrated the benefits of 11β-HSD1 inhibition, which patients are most likely to benefit? Importantly, the beneficial effects of 11β-HSD1 inhibition are not predicated on whether increased cortisol intracellular concentration in fat or liver is a

pathophysiological mechanism in the disease in question. Evidence of potent effects of 11β-HSD1 inhibition in healthy animals and humans described above illustrates that the benefits of this strategy are unlikely to be limited to patients with pathological up-regulation of 11β-HSD1 enzyme activity. Nevertheless, data above also emphasises that findings in healthy patients may not always be extrapolated to disease states of interest, and that the beneficial effects of 11β-HSD1 inhibition in patients may differ according to basal 11β-HSD1 activity in different tissues.

27. Glucocorticoid excess and deficiency produce the dramatic clinical features of Cushing's syndrome and Addison's disease, respectively. It has long been suspected that glucocorticoids contribute to the pathophysiology of more common disorders, including hypertension, obesity and type 2 diabetes mellitus. Understanding the physiological role of 11β-HSDs has led clinical investigators to address the importance of pre-receptor metabolism. Subtle deficiency of 11β-HSD2 may be important in some patients with essential hypertension [64;65]. The potential importance of 11β-HSD1 in pathophysiology and treatment has only been appreciated relatively recently. Impaired 11β-HSD1 may be important in liver. As above, in the leptin resistant Zucker obese rat, 11β-HSD1 is impaired in liver, a change predicted to ameliorate the local intrahepatic metabolic consequences of the obesity [52]. However this may also activate the hypothalamic-pituitary-adrenal (HPA) axis to compensate for the increased clearance of glucocorticoids though reduced hepatic regeneration (*see* the attached manuscript of Harris et al., "Intracellular regeneration of glucocorticoids by 11β-HSD1 plays a key role in regulation of the HPA axis: analysis of 11β-HSD1 deficient mice"). It appears that similarly impaired hepatic 11 β-HSD1 in liver occurs in patients with polycystic ovary syndrome [66] and primary obesity [53;53;54]. This predicts that hepatic 11β-HSD1 inhibition may be less beneficial in these patients.

28. However, enhanced 11β-HSD1 may also be important in increasing local glucocorticoid action and promoting adverse metabolic effects. In the face of impaired 11β-HSD1 activity in liver, Zucker obese rats show selectively enhanced activity of 11β-HSD1 in omental adipose tissue [52]. Very recent studies suggest the same tissue-specific pattern of dysregulation of 11β-HSD1 (i.e. impaired in liver, enhanced in adipose tissue) in human obesity[54] and type 2 diabetes mellitus (Figure 1). This predicts greater benefit to adipose metabolism from 11β-HSD1 inhibition in these patients. Accordingly, it is respectfully submitted that it is readily apparent that the present invention is applicable to, and can be practiced without

undue experimentation in, pathological syndromes involving adipose tissue such as obesity, as well as diabetes, and Cushing's syndrome.

29. While 11β-HSD1 activity has yet to be directly measured in the brain in pathological syndromes such as cognitive dysfunction of aging and depression, there is indirect evidence of the importance of cortisol in many patients with these conditions. For example, inhibition of cortisol secretion is an effective treatment in depression[69], and circulating levels of cortisol predict the rate of cognitive decline in older subjects [67;68]. Note again the attached manuscript of Yau et al. Accordingly, it is respectfully submitted that it is readily apparent that the present invention is applicable to, and can be practiced without undue experimentation in, pathological syndromes involving neuronal tissue, such as cognitive dysfunction of aging and depression. Note again the attached manuscript of Harris et al. (and references 61, 62 and 63) which confirms that 11β-HSD1 expression is highest in the hippocampus and cerebellum, where glucocorticoids have important effects upon electrophysiological parameters, behavior and cognition, as well as neuronal development, structure and survival.

30. These utilities of the present invention support the subject matter of claims 32-34 and the claims dependent thereon; and, the present application provides a written description and enablement for the subject matter of claims 32-34 and the claims dependent thereon. For instance, with respect to these syndromes and other conditions, a method for inhibiting reductase activity of 11-Beta-hydroxysteroid dehydrogenase 1 (11-Beta HSD1) in an animal in need thereof in adipose or neuronal tissue of the patient comprising administering to the animal an inhibitor of said reductase activity of 11-Beta HSD1 in an amount effective to so inhibit the reductase activity of 11-Beta HSD1, is quite useful; and, inhibiting 11-Beta HSD1 can be practiced without any undue experimentation from the disclosure in the present application and the knowledge in the art. Likewise, as to these syndromes and other conditions, a method for reducing intracellular glucocorticoid concentrations in an animal in need thereof in neuronal or adipose tissue comprising inhibiting the reductase activity of 11-Beta-hydroxysteroid dehydrogenase 1 (11-Beta HSD1) in said tissue, is quite useful; and, inhibiting 11-Beta HSD1 can be practiced without any undue experimentation from the disclosure in the present application and the knowledge in the art. Similarly, a method for determining whether a compound or composition is a regulator of intracellular glucocorticoid activity in adipose or neuronal tissue comprising determining whether said compound or composition inhibits

reductase activity of 11-Beta-hydroxysteroid dehydrogenase 1 (11-Beta HSD1) in said tissue, e.g., to ascertain new inhibitors of the enzyme in these tissues and/or to test whether known inhibitors act as they would be expected to act in view of the knowledge in the art and the disclosure in the present application, such practiced without any undue experimentation from the disclosure in the present application and the knowledge in the art. (*See also* discussion *infra* with respect to the Section 112 rejections.)

Further response to issues raised in the Office Action

31. It is respectfully submitted that the foregoing shows the patentability of the claimed subject matter. More in particular, as to the rejections of claims under 35 U.S.C. §102(b) as anticipated by Walker or Stewart, and under 35 U.S.C. §103 as obvious over Walker in view of Goodman and Gilman, it is respectfully submitted that the foregoing shows that one could not have extrapolated from Walker or Stewart to the present invention; and, that Stewart and Walker, either individually or in any combination, including in combination with Goodman and Gilman, fails to teach or suggest the present invention.[e] Therefore, reconsideration and withdrawal of the art rejections under Sections 102 and 103 are respectfully requested.[f]

32. With respect to the rejections under Sections 112, initially it is noted that the present application indeed shows in the Examples, and in reference 22 (Rajan, Edwards, & Seckl, J. Neurosci 16:65-70 (1996), copy attached), the neuronal effects of 11β-reductase inhibitors, as well as the role of 11β-HSD1 in adipocyte maturation, i.e., the reductase activity of 11β-HSD1 in adipose and neuronal tissue, and the benefits of inhibiting this activity. Furthermore, the Figures provide doses of an inhibitor of the reductase activity of 11-Beta HSD1 from which the skilled artisan can make and use the claimed invention, without undue

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[e] Goodman and Gilman are documents that contain general teachings that have already been discussed during the prosecution of the present application. These documents do not supply the deficiencies of Stewart and Walker. For instance, Goodman and Gilman do not allow one to extrapolate from the teachings to Stewart and Walker to adipose and neuronal tissue as in the present invention. Accordingly, it is respectfully submitted that Goodman and Gilman do not add to Stewart and Walker and that Stewart and Walker, either individually or in any combination, including in combination with Goodman and Gilman, fails to teach or suggest the present invention.

[f] It is also noted that claims 32 and 33 use the term "in an animal in need thereof"; and, we are advised and therefore believe that this phrase avoids having the claims read upon any previous administration of an inhibitor of the reductase activity of 11-Beta HSD1 for purposes other than inhibiting the reductase activity of 11-Beta HSD1 in adipose or neuronal tissue or reducing intracellular glucocorticoid concentration in neuronal or adipose tissue, i.e., we are advised and therefore believe that this phrase is used to have the claims avoid reading on any prior administrations of inhibitors of the reductase activity of 11-Beta HSD1. In other words, we are advised and therefore believe that the phrase is employed to further distinguish the claimed invention.

experimentation. Even further still, as to inhibitors of 11-Beta HSD1, the attached article by Monder and White, in Table IV at pages 196-198 provides a rather lengthy list of inhibitors of 11 β-hydroxysteroid dehyrogenase, such that contrary to the Office Action, the skilled artisan understands compounds encompassed by the phrase "an inhibitor of 11β-reductase" and this phrase is not indefinite or lacking clarity. Similarly, the term "an inhibitor of said reductase activity of 11-Beta HSD1" is clear and definite and the skilled artisan requires no undue experimentation to ascertain and administer an inhibitor of the reductase activity of 11-Beta HSD1 from the knowledge in the art and the teachings in the application.

33. Indeed, in addition to the lengthy list of inhibitors in Monder and White, we note that documents cited in the Office Action or during the prosecution also show inhibitors and modes of administration, such as Walker et al., "Carbenoxolone Increases Hepatic Insulin Sensitivity in Man: A Novel Role for 11-oxosteroid Reductase in Enhancing Glucocorticoid Receptor Activation," J. Clin. Endocrinology and Metabolism 80 (11): 3155-59 (1995). Thus, in the art, carbenoxolone and the lengthy list in Monder and White were known inhibitors. Gomez-Sanchez et al., "Central hypertensinogenic effects of glycyrrhizic acid and carbenoxolone," Am J Physiol 263 (6 Pt 1): E1125-E1130 (1992) showing that licorice, glycyrrhizic acid, and carbenoxolone were known inhibitors, as well as the infusion of glycyrrhizic acid and carbenoxolone into the lateral ventricle of the brain of the rat at doses less than that which has an effect when infused subcutaneously, produces hypertension, showing that such compounds were administered subcutaneously, orally, and by infusion; *see also* Whorwood et al., "Licorice inhibits 11 beta-hydroxysteroid dehydrogenase messenger ribonucleic acid levels and potentiates glucocorticoid hormone action," Endocrinology 132 (6): 2287-92 (1993) (copy of Abstract attached). Even further still, Homma et al., "A Novel 11β-Hydroxsteroid Dehydrogenase Inhibitor Contained in Saiboku-To, a Herbal Remedy for Steroid-dependent Bronchial Asthma," J. Pharm Pharmacol 46:305-309 (1994) (copy attached), Zhang et al., "Inhibition of 11β-Hydroxysteroid Dehydrogenase Obtained from Guinea Pig Kidney by Furosemide, Naringenin and Some Other Compounds," J Steroid Biochem Molec Biol 49(1):81-85 (1994) (copy attached), and Lee et al., "Grapefruit juice and its flavenoids inhibit 11β-hydroxysteroid dehydrogenase," Clin Pharmacol Ther 59:62-71 (1996) (copy attached), evince even more inhibitors that can be administered in known ways (both in terms of doses and routes of administration), such as flavenoids, which "are sold in tablet form in health food stores and drug

stores," and herbs or constituents of herbs. *See also* Morris et al., "Endogenous 11 beta-hydroxysteroid dehydrogenase inhibitors and their role in glucocorticoid Na+ retention and hypertension," Endocr Res 22(4):793-801 (1996) (progesterone metabolites as inhibitors, and progesterone is also a substance that can be administered – both in terms of doses and routes of administration - without undue experimentation).

34. Accordingly, the Examiner is respectfully requested to reconsider and withdraw the Section 112 rejections: The new claims do not specifically recite any particular method of treatment, i.e., no particular disease is being "treated"; the terms in the claims are indeed clear and definite; the present application contains a written description and enablement for the now claimed methods, and these methods clearly have utility; the now claimed methods are indeed operative, as discussed in the present application and herein and the attachments hereto; and, one skilled in the art, from the knowledge in the art and the teachings in the application, can practice the now claimed methods, without any undue experimentation, including without any undue experimentation in selecting a suitable inhibitor, and a dose therefore and a route of administration thereof.[g]

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[g] In this regard, we are advised and therefore believe that a specification need only begin teaching where the prior art leaves off. Thus, the present application did not need to provide an exhaustive list in inhibitors, doses of inhibitors, and routes of administration. Furthermore, in view of the inability to predict from teachings concerning the activity of 11β-HSD1 in the liver, the activity of 11β-HSD1 as a reductase in adipose and neuronal tissue, and thus the inability to predict the activity of an inhibitor of 11β-HSD1 in adipose and neuronal tissue from teachings concerning 11β-HSD1 in the liver, prior administrations of inhibitors of 11β-HSD1 did not teach or suggest the present invention. Indeed, we are advised and therefore believe that U.S. case law supports this assertion. For instance, we are advised and therefore believe that *In re Marshall,* 578 F.2d 301, 198 U.S.P.Q. (C.C.P.A. 1978) dealt with method claims for orally administering a compound in amounts sufficient to produce body weight loss. Some specific claims recited a range of the amount to be taken daily for producing weight loss, and the prior art, the Physician's Desk Reference (PDR) disclosed the same compounds for oral administration for treating ulcers and other gastrointestinal problems. We understand that the C.C.P.A. reversed the art rejection, stating that, "If anyone ever lost weight by following the PDR teachings, it was an unrecognized accident. **An accidental or unwitting duplication of an invention cannot constitute an anticipation.** *In re Felton,* 484 F.2d 495, 500, 179 U.S.P.Q. 295, 298 (C.C.P.A. 1983)" (emphasis added). *See also In re Felton* (cited by the C.C.P.A. in *Marshall, supra*); *Tilghman v. Proctor,* 102 U.S. 707 (1880); *Eibel Process Co. v. Minnesota and Ontario Paper Co.,* 261 U.S. 45 (1923) (all, as we are advised, for the proposition that the mere fact that a reference's generic disclosure might fortuitously result in narrower limitations of a later claim cannot constitute anticipation). **Moreover, we understand that even if a reference disclosure inevitably produces the claimed subject matter, if it is produced in such a manner as to go unnoticed and undetected, the reference cannot constitute an anticipation under Section 102.** *In re Seaborg (I),* 328 F.2d 993, 140 U.S.P.Q. 659 (C.C.P.A. 1964); *In re Seaborg (II),* 328 F.2d 996, 140 U.S.P.Q. 662 (C.C.P.A. 1964). *See also The General Tire & Rubber Co. v. Jefferson Chem. Co., Inc.,* 182 U.S.P.Q. 70 (2d Cir. 1974), *In re Antonson,* 124 U.S.P.Q. 132 (C.C.P.A. 1959), *International Nickel Co. v. Ford Motor Co.,* 119 U.S.P.Q. 72 (S.D.N.Y. 1958), and *Eibel Process Co. v. Minnesota & Ontario Paper Co.,* 261 U.S. 45 (1923) (all, as we are advised, for the classic holding that invention resides in recognizing a problem, as well as providing a solution). **Indeed, we understand that in *Eibel* the US Supreme Court recognized that accidental results, not intended and not appreciated, do not constitute anticipation.** *See also General Tire,* 182 U.S.P.Q. at

SUMMARY AND REQUEST FOR INTERVIEW

35. This declaration reviews the evidence that predominance of the 11β-reductase activity of 11β-HSD1 cannot be extrapolated from one tissue to another, that within each tissue the influence of the enzyme differs in different groups of patients, and therefore that the effect of 11β-HSD1 inhibition was not predictable in every tissue based on existing knowledge of its role in liver in 1995. This declaration also shows that the now claimed subject matter is sufficiently described and enabled in the present application, is operative, is claimed in clear and definite terms, and can be practiced without any undue experimentation. Accordingly, it is respectfully requested that the rejections of the September 1, 2000 Office Action be reconsidered and withdrawn. Moreover, we would welcome the opportunity to further explain any aspect of the present invention or this declaration to the Examiner, her SPE, and a Group 1600 Practice Specialist, in person. Therefore, if any issue remains as an impediment to allowance, we respectfully request a personal interview with the Examiner, her SPE, and a Group 1600 Practice Specialist, prior to issuance of any paper other than a Notice of Allowance; and, pursuant to this request the Examiner is also asked if she could please contact our representative, Mr. Thomas J. Kowalski, FROMMER LAWRENCE & HAUG LLP, 745 Fifth Avenue, New York, NY 10151, tel 212-588-0800, fax 212-588-0500, email TKowalski@FLHLaw.com, to arrange a mutually convenient time and manner for such an interview.

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76. In *International Nickel,* we are advised, the Court stated (with emphasis): **"Where the allegedly anticipating product was produced by chance and never recognized nor appreciated, one who later discovers and recognizes the product may patent it."** 119 U.S.P.Q. at 80. In the present case, it is we who recognized that 11β-HSD1 is a reductase in adipose and neuronal tissue, and that there can be beneficial results in inhibiting 11β-HSD1 in adipose and neuronal tissue. The prior art did not recognize or appreciate our invention; and thus, the prior art fails to teach or suggest our invention.

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33. Bland R, Worker CA, Noble BS, et al. Characterization of 11beta-hydroxysteroid dehydrogenase activity and corticosteroid receptor expression in human osteosarcoma cell lines. **J.Endocrinol.** 1999; **161**: 455-464.

34. Irony I, Cutler GB. Effect of carbenoxolone on the plasma renin activity and hypothalamic-pituitary-adrenal axis in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. **Clin.Endocrinol.(Oxf.)** 1999; **51**: 285-291.

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42. Rajan V, Edwards CRW, Seckl JR. 11β-Hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity. **J.Neurosci.** 1996; **16**: 65-70.

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55. Andrews RC, Andrew R, Herlihy O, Walker BR. Abnormal glucocorticoid metabolism and sensitivity in type 2 diabetes mellitus. **J.Endocrinol.** 1999; **163**: P28(Abstract)

56. Brem AS, Bina RB, Hill N, Alia C, Morris DJ. Effects of licorice derivatives on vascular smooth muscle function. **Life Sci.** 1997; **60**: 207-214.

57. Monder C, Miroff Y, Lakshmi V. 11 beta-hydroxysteroid dehydrogenase of rat testis. **Programme of the 73rd Meeting of the Endocrine Society** 1991; 1406(Abstract)

58. Walker BR, Connacher AA, Lindsay RM, Webb DJ, Edwards CRW. Carbenoxolone increases hepatic insulin sensitivity in man: a novel role for 11-oxosteroid reductase in enhancing glucocorticoid receptor activation. **J.Clin.Endocrinol.Metab.** 1995; **80**: 3155-3159.

59. Jamieson PM, Nyirenda MJ, Walker BR, Chapman KE, Seckl JR. Interactions between oestradiol and glucocorticoid regulatory effects on liver-specific glucocorticoid-inducible genes: possible evidence for a role of hepatic 11ß-hydroxysteroid dehydrogenase type 1. **J.Endocrinol.** 1998; **160**: 103-109.

60. Kotelevtsev YV, Holmes MC, Burchell A, et al. 11β-Hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid inducible responses and resist hyperglycaemia on obesity and stress. **Proc.Natnl.Acad.Sci.USA** 1997; **94**: 14924-14929.

61. Moisan M-P, Seckl JR, Edwards CRW. 11β-Hydroxysteroid dehydrogenase bioactivity and messenger RNA expression in rat forebrain: localization in hypothalamus, hippocampus and cortex. **Endocrinology** 1990; **127**: 1450-1455.

62. Moisan M-P, Seckl JR, Monder C, Agarwal AK, White PC, Edwards CRW. 11β-Hydroxysteroid dehydrogenase mRNA expression, bioactivity and immunoreactivity in rat cerebellum. **Neuroendocrinology** 1990; **2**: 853-858.

63. Lakshmi V, Sakai RR, McEwen BS, Monder C. Regional distribution of 11β-hydroxysteroid dehydrogenase in rat brain. **Endocrinology** 1991; **128**: 1741-1748.

64. Walker BR, Stewart PM, Shackleton CHL, Padfield PL, Edwards CRW. Deficient inactivation of cortisol by 11β-hydroxysteroid dehydrogenase in essential hypertension. **Clin.Endocrinol.(Oxf.)** 1993; **39**: 221-227.

65. Lovati E, Ferrari P, Dick B, et al. Molecular basis of human salt sensitivity: the role of the 11β-hydroxysteroid dehydrogenase type 2. **J.Clin.Endocrinol.Metab.** 1999; **84**: 3745-3749.

66. Rodin A, Thakkar H, Taylor N, Clayton R. Hyperandrogenism in polycystic ovary syndrome: evidence of dysregulation of 11beta-hydroxysteroid dehydrogenase. **N.Engl.J.Med.** 1994; **330**: 460-465.

67. Lupien S, Lecours AR, Lussier I, Schwartz G, Nair NPV, Meaney MJ. Basal cortisol levels and cognitive deficits in human aging. **J.Neurosci.** 1994; **37**: 2893-2903.

68. Lupien SJ, de Leon M, de Santi S, et al. Cortisol levels during human aging predict hippocampal atrophy and memory deficits. **Nature Neuroscience** 1998; **1**: 69-73.

69. Murphy BEP 1991 Treatment of major depression with steroid suppressive drugs. **Journal of Steroid Biochemistry and Molecular Biology** 39 121-126.

37. We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 26th Jan 2001 By: _____
Professor Jonathan R. Seckl

Dated: 26th January 2001 By: _____
Dr. Brian R. Walker

Dated: 26th January 2001 By: _____
Professor Christopher R.W. Edwards

CURRICULUM VITAE

Jonathan Robert **SECKL** BSc, MBBS, MRCP (UK), PhD, FRCPE, FMedSci

AWARDS, PRIZES AND FELLOWSHIPS

1978 Filliter Prize (1st in Pathology and Microbiology MB).
1980 Hons Viva (Medicine).
1980 Magrath Scholarship/Fellowes Gold Medal (Medicine MB).
1980 The Achison Exhibition (Medicine).
1984 Sir Jules Thorn Trust Research Fellowship.
1989 Wellcome Trust/Royal Society of Edinburgh Senior Clinical Research Fellowship.
1993 Wellcome Trust Senior Research Clinical Fellowship Renewal
1993 FRCP Edin
1994 Norage Pharmacia Prize (best paper on brain aging)
1998 Society for Endocrinology Medal
1999 Mortyn Jones Memorial Lecturer
1999 Fellowship, Academy of Medical Sciences

PRESENT APPOINTMENTS

1997 Moncrieff-Arnott Professor of Molecular Medicine, University of Edinburgh.
1995 Chairman, Molecular Medicine Centre, University of Edinburgh.
1989 Honorary Consultant Physician (Endocrinology), Western General Hospital.

PREVIOUS APPOINTMENTS

1996-97 Professor of Endocrinology, University of Edinburgh.
1993-96 Senior Lecturer in Medicine, University of Edinburgh.
1989-97 Wellcome Trust/Royal Society of Edinburgh Senior Clinical Research Fellow.
1987-92 Visiting Scientist, MRC Brain Metabolism Unit, Edinburgh.
1987-89 University of Edinburgh, Department of Medicine, Lecturer in Medicine
1984-87 Charing Cross and Westminster Medical School,Research Fellow Neuroendocrinology.

EDITORIAL BOARDS

Endocrinology (US); Steroids (US); Journal of Neuroendocrinology; Journal of Endocrinology

KEY RELEVANT PRIMARY PUBLICATIONS IN PEER-REVIEWED JOURNALS (OF 155)

Moisan M-P, **Seckl JR** and Edwards CRW (1990). 11ß-hydroxysteroid dehydrogenase mRNA expression and activity in rat hypothalamus, hippocampus and cortex. *Endocrinology* 127: 1450-1455.

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Brown RW, Chapman, KE, Edwards CRW and **Seckl JR** (1993). Human placental 11ß-hydroxysteroid dehydrogenase: partial purification of and evidence for a distinct NAD-dependent isoform. *Endocrinology* 132: 2614-2621.

Low SC, Assaad SN, Rajan V, Chapman KE, Edwards CRW and **Seckl JR** (1993). Regulation of 11ß-hydroxysteroid dehydrogenase by sex steroids in vivo: further evidence for the existence of a second dehydrogenase in rat kidney. *J Endocrinol* 139: 27-35.

Leckie C, Chapman KE, Edwards CRW and **Seckl JR** (1995). LLC-PK$_1$ cells model 11ß-hydroxysteroid dehydrogenase type 2 regulation of glucocorticoid access to renal mineralocorticoid receptors. *Endocrinology* 136: 5561-5569.

Rajan V, Edwards CRW, **Seckl JR** (1996). 11ß-hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity.*J Neuroscience* 16: 65-70.

Brown RW, Chapman KE, Edwards CRW and **Seckl JR** (1996). Purification of 11ß-hydroxysteroid dehydrogenase type 2 from human placenta. *Biochem J* 313: 997-1005

Brown RW, Kotolevtsev Y, Leckie C, Lindsay RS, Lyons V, Murad P, Mullins JJ, Chapman KE, Edwards CRW and **Seckl JR** (1996). Isolation and cloning of human placental 11ß-hydroxysteroid dehydrogenase-2 cDNA. *Biochem J* 313: 1007-1017

Brown RW, Diaz R, Robson AC, Kotolevtsev Y, Mullins JJ, Kaufman MH and **Seckl JR** (1996). The ontogeny of 11ß-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. *Endocrinol* 137: 794-797.

Voice M, **Seckl JR** and Chapman KE (1996). The sequence of 5'-flanking DNA from mouse 11ß-hydroxysteroid dehydrogenase type 1 and analysis of puative transcription factor binding sites. *Gene* 181: 233-235.

Lindsay RS, Lindsay RM, Edwards CRW and **Seckl JR** (1996). Inhibition of 11ß-hydroxysteroid dehydrogenase in pregnant rats and the programming of blood pressure in the offspring. *Hypertension* 27: 1200-1204.

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Waddell B, Benediktsson R and **Seckl JR.** (1996). 11ß-hydroxysteroid dehydrogenase type 2 in the rat corpus luteum: induction of mRNA expression and bioactivity coincident with luteal regression. *Endocrinology* 137: 5386-5391.

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Meaney MJ, Diorio J, Francis D, Weaver S, Yau JLW, Chapman KE, **Seckl JR** (2000). Postnatal handling increases the expression of cAMP-inducible transcription factors in the rat hippocampus: The effects of thyroid hormones and serotonin. *J Neurosci* 20: 3926-35.

Welberg LAM, **Seckl JR** and Holmes MC (2000). Inhibition of 11ß-hydroxysteroid dehydrogenase, the feto-placental barrier to maternal glucocorticoids, permanently programs amygdala glucocorticoid receptor mRNA expression and anxiety-like behavior in the offspring. *Europ J Neurosci* 12: 1047-1054.

Jamieson PM, Chaman KE, Walker BR and **Seckl JR** (2000). 11ß-hydroxysteroid dehydrogenase type 1 is a predominant 11ß-reductase in the intact perfused rat liver. *J Endocrinol* 165: 685-692.

Williams LJS, Lyons V, MAcLeod I, Rajan V, Darlington GJ, Poli V, **Seckl JR** and Chapman KE (2000). C/EBP□ regulates hepatic transcription of 11ß-hydroxysteroid dehydrogenase type 1; a novel mechanisms for cross-talk between the C/EBP and glucocorticoid signalling pathways. *J Biol Chem* 275: 30232-30239.

Harris HJ, Kotelevtsev Y, Mullins JJ, **Seckl JR** and Holmes MC (2001). 11ß-hydroxysteroid dehydrogenase type 1 null mice have altered hypothalamic-pituitary-adrenal axis activity: a novel control of glucocorticoid feedback. *Endocrinology* 142: 114-120.

REVIEWS AND CHAPTERS

Seckl JR (1993). 11ß-HSD isoforms and their implications for blood pressure regulation. *Eur J Clin Invest* 23: 589-601.

Seckl JR and Brown RW (1994). 11ß-hydroxysteroid dehydrogenase: on several roads to hypertension. *J Hypertens* 12: 105-112.

Seckl JR and Olsson T (1995). Glucocorticoids and the age-impaired hippocampus: cause or effect? *J Endocrinol* 145: 201-211.

Yau JLW and **Seckl JR** (1995). Corticosteroids and the brain. *Curr Opin Endocrinol Diabetes*: 2: 239-247.

Edwards CRW, Benediktsson R, Lindsay RS and **Seckl JR** (1996). 11beta-hydroxysteroid dehydrogenases: Key enzymes in determining tissue-specific glucocorticoid effects. *Steroids* 61: 263-269.

Seckl JR (1997). 11ß-hydroxysteroid dehydrogenase: regulator of glucocorticoid action in the brain. *Front Neuroendocrinol* 18: 49-99

Chapman KE, Kotelevtsev YV, Jamieson PM, Williams LJS, Mullins JJ and **Seckl JR** (1997). Tissue-specific modulation of glucocorticoid action by the 11beta-hydroxysteroid dehydrogenases. *Biochem Soc Trans* 25: 583-587.

Seckl JR and Chapman KE (1997). Medical and physiological aspects of the 11ß-hydroxysteroid dehydrogenase system. *Eur J Biochem* 249: 361-364.

Seckl JR and Nyirenda MJ (1999). Glucocorticoids, feto-placental 11ß-hydroxysteroid dehydrogenase and the programming of hypertension. *Handbook of Hypertension* Vol. 19: Development of the Hypertensive Phenotype; McCarty R, Blizard DA, Chevalier RL (eds); Elsevier, Amsterdam, pp103-136.

Seckl JR (2000). 11ß-hydroysteroid dehydrogenases. *Encyclopaedia of Stress*. Fink G (ed). (in press).

Seckl JR and Walker BR (2001). 11ß-hydroxysteroid dehydrogenase type 1: a tissue-specific amplifier of glucocorticoid action. *Endocrinology* (in press).

Seckl JR and Walker BR (eds) (2001). Steroid Metabolism (book). *Bailliere's Clinical Endocrinology and Metabolism* (in press).

CURRICULUM VITAE **Dr Brian Robert Walker** **DOB 12/7/63 British**

Degrees etc

1984	BSc (1st class Hons)	Immunology	University of Edinburgh
1986	MB ChB		University of Edinburgh
1989	MRCP (UK)		
1993	MD (with Distinction)		University of Edinburgh
1999	FRCP Edinburgh		

Current Appointments

British Heart Foundation Senior Research Fellow (since Nov 1996)
Honorary Consultant Physician, Western General Hospital (since Nov 1996)
Member of Scientific Advisory Board, Wellcome Trust Cardiovascular Research Initiative and Centre for Cardiovascular Biology, University of Edinburgh (since Aug 1998)
Associate Director, Clinical Research Centre, University of Edinburgh (since June 1999)
Reader in Medicine, University of Edinburgh (since Oct 1999)
Director, GCMS Core Laboratory, Wellcome Trust Clinical Research Facility, Edinburgh (since Dec 1999)

Previous Appointments

1993-6	Lecturer in Medicine	University of Edinburgh
1992-3	Sir Stanley Davidson Lecturer in Medicine	University of Edinburgh
1989-93	MRC Training Fellow	University of Edinburgh
1987-89	SHO Rotation	Western Infirmary Glasgow

Postgraduate Prizes

William Leslie Prize for research awarded by University of Edinburgh Faculty of Medicine, 1991
Shortlisted for Young Investigator Award at the British Hypertension Society, Dublin, 1991
Finalist in Medical Research Society Young Investigator Prize competition, London, 1993
Wilfrid Card Lectureship and Medal, Edinburgh, 1994
Young Endocrinologist Award at the British Endocrine Societies, Warwick, 1995
Poster Prize at the Society for Endocrinology, London, 1995
Young Investigator Award at the International Society of Hypertension, Glasgow, 1996
Merck Senior Fellow Award at the International Congress of Endocrinology, San Francisco, 1996
British Hypertension Society Cardiovascular Research Travelling Fellowship to visit University of Umea, Sweden, 1997
Young Investigator Award at the British Hypertension Society, Bristol, 1997
Short-listed for the Austin Doyle Award at the International Society for Hypertension, Amsterdam, 1998
Special Travel Award to the International Society for Hypertension, Chicago, 2000

Academic recognition and activities

Member of Editorial Board for Clinical Endocrinology 1999-
Senior Editor for Journal of Endocrinology 2000-

Refereed grant applications for British Heart Foundation, Wellcome Trust, Medical Research Council, and British Diabetic Association; reviewed numerous manuscripts for diverse journals; examined 5 postgraduate theses

In year 2000, invited to lecture in Umea (Sweden), Gothenburg (Sweden), Phoenix (USA), Monte Carle (Monaco), and Nice (France); also lecturer at British Endocrine Societies Joint Meetings in 1998 and 1999, and at Association of Clinical Biochemists in Glasgow 1998.

RELEVANT PUBLICATIONS

Peer-Reviewed Publications

Dr Walker is author of more than 50 peer-reviewed articles since 1990. The following are especially relevant to 11β-hydroxysteroid dehydrogenases:

1. Walker BR, Yau JL, Brett LP, Seckl JR, Monder C, Williams BC, Edwards CRW (1991) 11ß-Hydroxysteroid dehydrogenase in vascular smooth muscle and heart: implications for cardiovascular responses to glucocorticoids. *Endocrinology*, 129: 3305-3312.
2. Walker BR, Edwards CRW (1991) 11ß-Hydroxysteroid dehydrogenase and enzyme-mediated receptor protection: Life after liquorice? *Clinical Endocrinology*, 35: 281-289.
3. Walker BR, Connacher AA, Webb DJ, Edwards CRW (1992) Glucocorticoids and blood pressure: a role for the cortisol/cortisone shuttle in the control of vascular tone in man. *Clinical Science*, 83: 171-178.
4. Walker BR, Moisan M-P (1992) Multiple isoforms of the cortisol-cortisone shuttle. *Journal of Endocrinology*, 133: 1-3.
5. Walker BR, Campbell JC, Williams BC, Edwards CRW (1992) Rapid Communication: Tissue-specific distribution of the NAD^+-dependent isoform of 11ß-hydroxysteroid dehydrogenase. *Endocrinology*, 131: 970-972.
6. Walker BR, Campbell JC, Fraser R, Stewart PM, Edwards CRW (1992) Mineralocorticoid excess and inhibition of 11ß-hydroxysteroid dehydrogenase in patients with ectopic ACTH syndrome. *Clinical Endocrinology*, 37: 483-492.
7. Walker BR, Stewart PM, Shackleton CHL, Padfield PL, Edwards CRW (1993) Deficient inactivation of cortisol by 11ß-hydroxysteroid dehydrogenase in essential hypertension. *Clinical Endocrinology*, 39: 221-227.
8. Walker BR, Sang KS, Williams BC, Edwards CRW (1994) Direct and indirect effects of carbenoxolone on responses to glucocorticoids and noradrenaline in rat aorta. *Journal of Hypertension*, 12: 33-39.
9. Walker BR, Williams BC, Edwards CRW (1994) Regulation of 11ß-hydroxysteroid dehydrogenase activity by the hypothalamic-pituitary-adrenal axis in the rat. *Journal of Endocrinology*, 141: 467-472.
10. Walker BR, Aggarwal I, Padfield PL, Stewart PM, Edwards CRW (1995) Endogenous inhibitors of 11ß-hydroxysteroid dehydrogenase in hypertension. *Journal of Clinical Endocrinology and Metabolism*, 80: 529-533.

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1 Seckl JR, Walker BR (eds)(2000) Bailliere's Best Practice and Research in Clinical Endocrinology and Metabolism: Disorders of Steroid Metabolism. Bailliere Tindall, London, (in press).

Abstracts & Letters

Dr Walker is author of >130 published abstracts and letters.

1: *Clin Endocrinol (Oxf)* 1999 Jan;50(1):63-8 Related Articles, Books, LinkOut

Online

An in vivo study of the cortisol-cortisone shuttle in subcutaneous abdominal adipose tissue.

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OBJECTIVE: Previous in vitro studies have demonstrated significant 11-beta hydroxysteroid dehydrogenase (11 beta-HSD) oxo-reductase activity in visceral, but not subcutaneous adipose stromal cells. We have conducted an in vivo study of the cortisol-cortisone shuttle in subcutaneous abdominal adipose tissue. DESIGN: We measured arteriovenous (A-V) differences in serum cortisol and cortisone across subcutaneous abdominal adipose tissue and forearm muscle in a heterogeneous group of subjects. PATIENTS: We studied 34 subjects (male:female = 12:22), age median (interquartile range) 45 (19-65) years, body mass index 32.7 (20.4-77.1) kg m-2, total body fat 34.4 (5.6-119.1) kg. MEASUREMENTS: Serum cortisol and cortisone were measured in serum samples from a radial artery, superficial epigastric vein and deep forearm vein. Abdominal adipose and forearm blood flow rates were measured by 133Xenon washout and plethysmography, respectively. RESULTS: For cortisone, there was significant ($P < 0.001$) clearance by adipose tissue, with an A-V difference of 4 (1-7) nmol/l. For cortisol there was a trend for arterial concentrations (203 (142-292) nmol/l) to be lower than venous (225 (152-263) nmol/l), but this was not significant. The adipose tissue cortisone clearance rate correlated with total body fat ($r = 0.35$, $P = 0.05$). CONCLUSIONS: We have demonstrated 11 beta-HSD oxo-reductase activity in subcutaneous abdominal adipose tissue, which may be increased in obesity.

PMID: 10341857, UI: 99273403

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Endogenous 11 beta-hydroxysteroid dehydrogenase inhibitors and their role in glucocorticoid Na+ retention and hypertension.

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Related Resources

11 beta-hydroxysteroid dehydrogenase (11 beta-HSD) metabolizes active glucocorticoids to their inactive 11-dehydro products and protects renal mineralocorticoid receptors from the high circulating levels of endogenous glucocorticoids. 11 beta-HSD has been suggested to be important not only in the control of renal sodium retention but also blood pressure. We had previously shown that 11 alpha- and 11 beta-hydroxyprogesterone (11 alpha- and 11 beta-OHP) were (I) potent inhibitors of 11 beta-HSD (Isoforms 1 and 2) activity in vitro, (ii) able to confer mineralocorticoid (MC) activity upon corticosterone (B) in vivo and (iii) hypertensinogenic when chronically infused into Sprague-Dawley (SD) rats. In addition we also showed that 3 alpha,5B-tetrahydroprogesterone (3 alpha,5B-THP) and chenodeoxycholic acid (CDCA) were potent inhibitors of 11 beta-HSD1 activity but not 11 beta-HSD2 activity, however, these substances were still able to confer MC activity upon B in the adrenalectomized rat. To assess the possible blood pressure modulating effects of 3 alpha,5B-THP and CDCA we have now infused these substances into intact SD rats continuously for 14 days. Both 3 alpha,5B-THP and CDCA caused a significant elevation in blood pressure within seven days, an effect that persisted throughout the 14-day infusion. These results show that both 3 alpha,5B-THP and CDCA are hypertensinogenic in the rat and that the inhibition of either 11 beta-HSD2 or 11 beta-HSD1 activity by endogenous progesterone metabolites and CDCA may be involved in the pathology of hypertension.

PMID: 8969942, UI: 97124806

1: *Endocrinology* 1993 Jun;132(6):2287-92 Related Articles, Books

Licorice inhibits 11 beta-hydroxysteroid dehydrogenase messenger ribonucleic acid levels and potentiates glucocorticoid hormone action.

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11 beta-Hydroxysteroid dehydrogenase (11 beta HSD) is responsible for the interconversion of cortisol to cortisone [corticosterone (B) to 11-dehydrocorticosterone in rodents] and confers ligand specificity to the mineralocorticoid receptor. Inhibition of 11 beta HSD by licorice derivatives [glycyrrhizic and glycyrrhetinic (GE) acids] results in cortisol/B and not aldosterone acting as a potent mineralocorticoid. 11 beta HSD is ubiquitously expressed and, by converting active glucocorticoid to inactive metabolites, may be an important prereceptor regulator of ligand access to the glucocorticoid receptor (GR). To investigate this further, we have studied the effect of 11 beta HSD inhibition by licorice derivatives on PRL gene expression (a known glucocorticoid target gene) in rat pituitary GH3 cells. Glycyrrhizic acid administration to rats in vivo (75 mg/kg.day for 5 days) resulted in inhibition of 11 beta HSD activity, as previously reported, but also a significant reduction in steady state 11 beta HSD mRNA levels in both predominantly mineralocorticoid (kidney and distal colon) and glucocorticoid (liver and pituitary) target tissues. In vitro, 11 beta HSD mRNA and activity were present in rat pituitary GH3 cells (81% conversion of B to 11-dehydrocorticosterone/4 x 10(6) cells after 24-h incubation) and inhibited by GE in a dose-dependent fashion. While B or GE alone (10(-8)-10(-5) M) had little or no effect on PRL mRNA levels or immunoassayable PRL, combinations of GE plus B resulted in marked inhibition of PRL mRNA levels and secretion, to such an extent that a concentration of 10(-6) M B with 10(-6) M GE was more potent than equimolar concentration of the synthetic GR agonist RU 28362. This inhibitory effect on PRL mRNA levels was blocked by a 10-fold excess of the GR antagonist RU 38486, but not by a 10-fold excess of the mineralocorticoid receptor antagonist RU 26752, confirming that this potentiation of glucocorticoid hormone action was operating through the GR and not the mineralocorticoid receptor. In addition to its established role as a competitive inhibitor of 11 beta HSD, licorice results in pretranslational inhibition of 11 beta HSD both in vitro and in vivo. 11 beta HSD is clearly an important mechanism in regulating tissue

levels of active glucocorticoid and, hence, ligand supply to the GR.

PMID: 8504732, UI: 93279206

Lack of tissue glucocorticoid reactivation in 11ß-hydroxysteroid dehydrogenase type 1 knockout mice prevents age-related cognitive impairment.

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Abstract

11ß-hydroxysteroid dehydrogenase type 1 (11ß-HSD-1) intracellularly regenerates active corticosterone from circulating inert 11-dehydrocorticosterone (11-DHC) in specific tissues. We examined the functional significance of 11ß-HSD-1 in the CNS using knockout mice. Aged wild-type mice developed elevated corticosterone levels which correlated with learning deficits in the watermaze. In contrast, despite elevated plasma corticosterone levels throughout life, aged 11ß-HSD-1 knockout mice did not manifest deficits in watermaze learning, implicating lower intraneuronal corticosterone levels through lack of 11-DHC reactivation. Indeed, aged knockout mice showed significantly lower hippocampal tissue corticosterone levels than wild-type controls. Selective 11ß-HSD-1 inhibitors may protect against cognitive decline with age.

Introduction

Much evidence in rodents, non-human primates and humans suggests that prolonged elevation of blood glucocorticoid levels impairs cognitive function, an effect which becomes more marked with ageing [1-3]. Chronic glucocorticoid excess has deleterious actions upon brain neuronal biochemistry and electrophysiological function (particularly in the hippocampus by inhibiting the formation of long-term potentiation [4], reduces dendritic structural complexity [5] and may eventually produce neuronal loss [6,7]. Manipulations which keep glucocorticoid levels low throughout life (including adrenalectomy with low-dose glucocorticoid replacement, and neonatal handling [8,9]) prevent the emergence of cognitive deficits with age. However, such approaches are not appropriate for human therapy.

Recently, it has been recognised that tissue glucocorticoid concentrations are determined not only by plasma hormone levels, but also by intracellular 11ß-hydroxysteroid dehydrogenases (11ß-HSDs) which locally interconvert active glucocorticoids (corticosterone in rats and mice, cortisol in humans) and inert 11-keto forms (11-dehydrocorticosterone (11-DHC), cortisone[10]). The importance of such enzymic interconversion is clearly demonstrated by the action of 11ß-HSD type 2, an exclusive dehydrogenase, which potently inactivates glucocorticoids thus allowing selective access for aldosterone to intrinsically non-selective mineralocorticoid receptors (MR) in the kidney [11,12]. The observation of 11ß-HSD in neurons in the brain prompted suggestions that the enzyme might attenuate the deleterious effects of chronic glucocorticoid excess on neuronal function and survival [13]. However, the predominant isoform in the brain, 11ß-HSD type 1 (11ß-HSD-1), which is located in the membrane and lumen of the endoplasmic reticulum[14], appears to function as a predominant 11ß-reductase (regenerating active glucocorticoids from inert 11-DHC) in intact cells in primary culture [15], including neurons [16], and in the liver *in vivo* [17]. This reductase activity, far from protecting neurons against glucocorticoid excess, would be anticipated to increase intraneuronal glucocorticoid levels, potentiating neurotoxicity. Indeed, *in vitro*, otherwise inert 11-DHC potentiates kainate-induced neurotoxicity in hippocampal neurons in culture, an effect lost in the presence of an 11ß-HSD inhibitor [16]. If such regeneration of glucocorticoids within neurons is important *in vivo*, inhibition of 11ß-reductase might attenuate neuronal dysfunction and the

consequent cognitive decline with ageing. To test this hypothesis, we examined the effects of ageing on cognitive function in mice homozygous for targeted disruption of the 11ß-HSD-1 gene [17]; the tests used were a proximally-cued version of the Morris watermaze task and the Porsolt forced swim test [18].

Results

PERFORMANCE IN CUED PLATFORM WATERMAZE TASK.

During a 5 day training period, young (4-7 months old) wild type mice showed a progressive reduction in the latency to escape onto a flagged hidden platform in the watermaze (mean of the 4 trials per day; $F_{5,24}=5.3$, $P< 0.005$; FIG 1a). Although on initial entry into the pool (trial 1, day 1) the escape latency did not differ between the young and aged (18-20 months old) mice [35.8±5.2 s (all young); 34.0±4.9 s (all old)], the reduction in latency with training did not occur in the aged wild type controls as a group ($F_{7,32}=1.6$, $p=0.2$); their average escape latencies (trials 1-4) for each day of training were significantly longer than young wild type mice ($p < 0.05$). Young 11ß-HSD-1$^{-/-}$ mice performed as well as young wild type controls in the proximally-cued platform watermaze task (FIG 1a). Strikingly, aged 11ß-HSD-1$^{-/-}$ mice also learned the task well, showing decreased escape latencies to find the platform with increasing days of training ($F_{7,32}=17$, $P < 0.001$). The aged 11ß-HSD-1$^{-/-}$ mice performed as well as young wild type mice on the last 2 days of training.

The differences between aged wild type and 11ß-HSD-1 null mice in the cued platform watermaze task did not appear to be due to non-cognitive factors such as swim speeds or visual processing. Although the significantly longer escape latencies shown by the aged mice (both genotypes) at the end of the first day of training ($P<0.05$) (FIG 1a) may partly reflect a slower swim speed ($P<0.01$) [wild-type, 0.24m/s (young), 0.17m/s (old); 11ß-HSD-1$^{-/-}$, 0.22m/s (young), 0.14m/s (old)], the average swim speed did not vary significantly across the last 4 days of training for individual mice ($P=0.98$). Therefore changes of escape latency associated with consecutive training trials reflects learning of the task. Visual processing abilities can also affect learning, since a direct correlation between the severity of photoreceptor degeneration and performance of aged rats in the watermaze has been reported [19]. However, histopathological examination of the eyes of young and aged mice

from both genotypes showed normal retinal integrity (all had retinas with an outer nuclear layer composed of ~10 rows of photoreceptor nuclei, a 5 cell layer thick inner nuclear layer and a single cell layer of ganglion cells; not shown).

129/Ola WILD-TYPE MICE PERFORMANCE IN THE WATERMAZE FOLLOWING HIPPOCAMPAL LESIONS

To determine whether the proximally-cued platform watermaze task is hippocampus-dependent in 129/Ola mice, we selectively lesioned the whole hippocampus using ibotenate acid. Cresyl violet staining of brain sections confirmed selective removal of hippocampal cells with no evidence of damage to extrahippocampal areas. The swim speed and escape latency to find the platform on initial entry into the pool (trial 1, day 1) did not differ between the sham and hippocampal lesioned mice, but by the end of first day of testing (trial 4) and across the remaining days, the hippocampal lesioned mice showed significantly longer escape latencies than the sham controls ($P<0.001$). In contrast with the sham controls, hippocampal lesioned mice were unable to learn the task, showing no decrease in escape latency across the four days of training ($F_{4,20}=0.38$, $P=0.8$; FIG 1b).

PLASMA CORTICOSTERONE, 11-DEHYDROCORTICOSTERONE (11-DHC) AND CORTICOSTEROID BINDING GLOBULIN (CBG) LEVELS

Basal (morning) plasma corticosterone levels were low in young wild type controls, but became markedly elevated with age ($p<0.001$; FIG 2). Young 11ß-HSD-$1^{-/-}$ mice had significantly higher basal plasma corticosterone levels than young wild-type controls ($p<0.001$; FIG 2), confirming previous data [17]. There was no further rise of plasma corticosterone in 11ß-HSD-$1^{-/-}$ mice with age, so both genotypes had similarly elevated corticosterone levels in aged mice (FIG 2). In wild-type controls, plasma corticosterone levels correlated directly with the escape latency to find the flagged hidden platform (Table 1, FIG.3). There was no correlation between plasma glucocorticoid levels and watermaze performance in 11ß-HSD-$1^{-/-}$ mice (Table 1).

Plasma 11-DHC levels in the wild type mice correlated with plasma corticosterone levels ($r=0.65$, $p<0.02$). 11ß-HSD-$1^{-/-}$ mice had significantly elevated 11-DHC levels (which they cannot activate

to corticosterone [17]) ($P<0.05$, FIG 2). 11-DHC in 11ß-HSD-$1^{-/-}$ mice did not correlate with plasma corticosterone (FIG 2). Plasma CBG levels were not significantly altered by genotype or age (FIG 2).

DISTRIBUTION OF INFUSED [^{3}H]-LABELED CORTICOSTERONE IN BRAIN TISSUES OF WILD TYPE AND 11ß-HSD-1 KNOCKOUT MICE

To determine whether effective intracerebral glucocorticoid levels in the hippocampus were altered in aged 11ß-HSD-$1^{-/-}$ mice, [^{3}H]-corticosterone was infused under equilibrium conditions. The amount of [^{3}H]-corticosterone retained in the hippocampus was significantly lower in 18 month old 11ß-HSD-$1^{-/-}$ mice (64% decrease, $P<0.05$; FIG. 4). [^{3}H]-corticosterone levels were also lower in the brain stem (61% decrease, $P<0.05$) and showed a similar non-significant trend for lower levels in cerebellum. There were no significant differences between genotypes in cortex which expresses 11ß-HSD-1 only in a single layer [20] (FIG. 4).

PERFORMANCE IN PORSOLT FORCED SWIM TEST

To examine a distinct behavioural paradigm that may be sensitive to age-related decline, we carried out a forced swim test. This assesses ability to habituate to a stressful environment. All groups of mice (wild-type and 11ß-HSD-$1^{-/-}$, both young and old) spent a similar amount of time floating during an initial 5 min observation period (FIG. 5). The latency to immobility was reduced and the floating time increased in all mice when re-tested 24h later, indicating that the mice had learned to stop trying to escape and/or conserve energy. Compared with young controls, both aged wild-type and 11ß-HSD-$1^{-/-}$ mice showed similar, non-significant trends for poorer learning (28% and 24% decrease in floating times, respectively ($P>0.1$); FIG. 5). Basal plasma corticosterone levels did not correlate with performance in the forced swim test (wild-type mice, $r= -0.3$, $p=0.3$; 11ß-HSD-$1^{-/-}$ mice $r=0.5$, $p=0.15$). This learning behaviour therefore appears to be unassociated with glucocorticoids or 11ß-HSD-1 genotype in mice.

Discussion

Basal plasma corticosterone levels are low in young wild type mice, but rise with age in a proportion of animals, consistent with findings in rats and humans [2,21,22]. In mice, as in other species, elevated corticosterone levels correlate with specific age-associated cognitive impairments [1,3,23]. Corticosterone levels in 18 month old rats may predict spatial learning in the watermaze 6 months later (Yau & Seckl, unpublished data), emphasising again the causal link between corticosterone and cognitive function in individual animals and echoing analogous prospective data in humans [2]. The key question addressed here is whether the effects of active glucocorticoids are in any way influenced by the local conversion of inert circulating 11-dehydrocorticosterone (11-DHC) by the activity of 11ß-reductase within the CNS. The data suggest that regeneration of active corticosterone by 11ß-HSD-1 within the brain is indeed important, since glucocorticoid-associated cognitive deficits with ageing do not develop when the enzyme is absent.

In young wild type mice, 11-DHC levels are approximately half those of total corticosterone. However, around 95% of corticosterone is bound to plasma proteins (largely CBG [24]) and therefore unable to enter cells, so 'free' corticosterone levels are less than 11-DHC levels, which is little bound by CBG (see FIG 2). Thus in young wild type mice a substantial proportion of intracellular active glucocorticoid is likely to be derived from circulating 11-DHC, activated within cells to corticosterone by 11ß-HSD-1, the only murine enzyme catalysing 11ß-reduction [17]. Aged wild type mice have elevated plasma corticosterone levels, but 11-DHC is still likely to contribute substantially to intracellular corticosterone (see FIG 2). In contrast, although 11ß-HSD-1$^{-/-}$ mice have elevated 11-DHC levels, these cannot be converted to corticosterone within cells. Elevated plasma 11-DHC is presumably a consequence of reduced clearance due to loss of metabolism by 11ß-HSD-1. Increased plasma corticosterone levels in the null mice are likely to reflect both increased adrenal production to compensate for increased clearance (due to lack of regeneration by 11ß-HSD-1) and a loss of 11ß-reductase in CNS areas responsible for glucocorticoid negative feedback upon the hypothalamic-pituitary-adrenal (HPA) axis. Indeed, the hippocampus, hypothalamus and pituitary all express 11ß-HSD-1 [20,25]. Loss of glucocorticoid regeneration in such sites would be anticipated to attenuate feedback and thus increase HPA activity. Thus in the null mice the only source of

intracellular glucocorticoid is circulating corticosterone.

Strikingly, in the face of increased plasma levels of active corticosterone throughout life, the 11ß-HSD-1$^{-/-}$ mice resist glucocorticoid-associated hippocampal cognitive impairments with ageing, as shown in the flagged hidden platform watermaze task. This strongly suggests that 11ß-reductase regeneration of corticosterone from 11-DHC is an important determinant of effective intraneuronal glucocorticoid action. Plasma CBG levels were similar in 11ß-HSD-1$^{-/-}$ and wild type mice and were not significantly altered with ageing. As 5% of corticosterone is 'free' and assuming near complete conversion of 11-DHC to corticosterone by 11ß-HSD-1 in wild type neurons, then 'intraneuronal' corticosterone levels are likely to be lower in aged 11ß-HSD-1 null mice than in aged wild type controls, which have the additional burden of corticosterone regenerated from 11-DHC (see FIG 2). In support of this contention, retention of [^{3}H]-corticosterone in the hippocampus of aged 11ß-HSD-1$^{-/-}$ mice was less than half that of aged wild type controls, in the face of similar plasma corticosterone levels at this age. Taken together, the data suggest that 11ß-HSD-1 contributes substantially to active corticosterone levels within the hippocampus *in vivo* and hence to the deleterious effects of glucocorticoids with ageing.

All the mice failed to learn the classical hippocampus-dependent spatial task where the hidden platform is located using extra-maze distal cues around the experimental room (data not shown). Indeed, mice of the 129 strain generally perform poorly in the distally-cued version of the watermaze task [26,27]. The assumption that learning of the proximally-cued platform watermaze task is partly/largely hippocampus-independent is based on findings in rats. However, this interpretation may be species dependent, since hippocampal lesions in some strains of mice impair learning in both distally- and proximally-cued platform versions of the watermaze task [28]. Recent studies have shown that both spatial and non-spatial information can be encoded within the hippocampus in a manner consistent with the mnemonic demands of the task [29]. The severe impairment of the hippocampal lesioned 129/Ola mice to learn the proximally-cued platform watermaze task suggests hippocampal-dependence, at least in this strain.

In wild type mice, watermaze performance in the flagged hidden platform task correlated with glucocorticoid levels, consistent with previous data in the spatial learning watermaze task which also correlate with basal corticosterone levels [3]. Interestingly, this correlation is lost in 11ß-HSD-1$^{-/-}$ mice, presumably because local tissue corticosterone levels in the brain regions involved in the cognitive task are lower than in wild type controls due to the lack of 11-DHC reactivation and fail to reach the threshold for cognitive decline. This contention is supported by the requirement for clearly elevated glucocorticoid levels before inhibition of long-term potentiation or atrophy of hippocampal dendritic structure [5,30,31].

To determine whether there were more general changes in cognitive function in 11ß-HSD-1$^{-/-}$ mice, we used the forced swim task which examines coping strategy in an apparently hopeless situation [18]. The performance in this learning task did not correlate with corticosterone levels and was not affected by 11ß-HSD-1 genotype, suggesting that loss of 11ß-HSD-1 is selectively protective against glucocorticoid-associated cognitive impairments in aged mice. It remains to be determined whether hippocampal neuronal structural changes and other features of glucocorticoid excess with ageing are also prevented in the 11ß-HSD-1$^{-/-}$ mice. Non-selective 11ß-HSD inhibition increases hepatic insulin sensitivity in humans [32], in line with the hepatic phenotype of the 11ß-HSD-1$^{-/-}$ mouse [17]. Clearly, selective inhibitors of 11ß-reductase could act as useful agents to prevent cognitive decline with age. 11ß-HSD-1 represents a plausible therapeutic target to attenuate effective glucocorticoid action in the brain *in vivo*.

Methods

Male mice with a targeted disruption of the 11ß-HSD-1 gene were inbred on the original 129/Ola strain background as were wild type controls [17]. Young (4-7 months old) and aged (18-20 months old) mice were housed in individual cages on a 12 hour light-dark cycle (light on 7am to 7pm). Both genotypes had similar survival rates up to 23 months. All animals (n=28) were tested between 9am and 5pm. Animal care was in accordance with Home Office and institutional guidelines.

Watermaze - The watermaze was a circular tank (1.8 m diameter, 0.60 m height) filled to a depth of 35 cm with water (25±1°C) made opaque with latex liquid. The paths taken by the mice were monitored with an overhead video camera connected to an image analyzer (HVS Image, UK) and an Acorn Archimedes running software which sampled the co-ordinates on-line at 10Hz for subsequent automated data analysis. For all training trials, the computer calculated the escape latency and the average swim speed of the mice. The day before training, all mice were given a 60s swim and allowed to mount a 30cm platform submerged just below the water surface.

Mice were tested in the proximally-cued version of the maze where the platform position was indicated by a visible flag. This behavioural task requires the animal to associate the flag with escape from the water. The target platform was submerged just below the water surface and located under a flag (5cm high x 8cm wide) attached to the platform, 10cm above the water. Curtains were drawn around pool to exclude extra-maze cues. Training consisted of 4 trials per day for 5 days, each trial lasting until the mouse reached the platform (escape latency) or swam for 60s. After each trial the mice were allowed to remain on the platform for 30s. Mice were released in the pool from one of 4 equally spaced start positions along the perimeter of the pool in a predetermined, pseudorandom order. On the first day of training, a larger platform (30cm diameter) was used to increase the chance for the mice to find the platform. From the second day onward the diameter of the platform was 20cm. The location of the submerged platform was varied randomly from trial to trial. Mice were returned to drying cages and kept warm under a heat lamp between trials (inter-trial interval 10 min). The performance of all mice were videotaped in a single blind analysis. Mice which tended to float rather than swim were excluded.

Ibotenate lesions of hippocampus - Adult male 129/Ola mice (25g) were anaesthesized with Hypnorm/Hypnovel (0.8ml/kg, i.p.) and placed in a stereotaxic frame. Hippocampal lesions were made by 20 discrete injections of 0.03µl ibotenic acid (10mg/ml; Sigma, U.K) at 0.06µl/min; the needle was left in place for 2 min following injection to avoid spread of the toxin up the needle tract. Stereotaxic co-ordinates were those used by Jarrard [33], modified for mice. Sham operated controls underwent similar surgical procedures except that no drug was infused. Six weeks after surgery,

mice were tested in the cued version of the watermaze task, as described above. Following behavioural testing, mice were anaesthetised and perfused intracardially with saline and 4% paraformaldehyde. The brains were stored in the perfusate for 1 week, then frozen, sectioned coronally (30μm) and stained with cresyl violet to determine cell loss.

Porsolt forced swim test - Mice were forced to swim in a large clear beaker (2L) filled with water (8cm deep, 25°C) from which they could not escape [18]. The total time spent floating (immobility) was measured over 5min on two mornings separated by 24 h. The mice were videotaped and data analysis was performed blind to genotype or age.

Corticosterone, 11-DHC and CBG measurements - Blood samples were obtained by cardiac puncture immediately after killing mice by cervical dislocation in the morning. Plasma corticosterone levels were measured by radioimmunoassay [34] modified for microtitre plate scintillation proximity assay. The intra- and inter-assay coefficients of variation were 9.4% and 9.2%, respectively. Plasma 11-dehydrocorticosterone (11-DHC) was measured by the above radioimmunoassay but using [^{3}H]-11-DHC (synthesised using kidney homogenates, NAD and [^{3}H]-corticosterone [35]) and the antibody for 11-DHC.

For the measurement of CBG, endogenous steroids were removed by diluting plasma samples 1:100 in dextran coated charcoal in phosphate buffered saline-gelatin (DCC-G) and incubation for 1 hr at RT followed by centrifugation at 3000g for 15min. Aliquots (100µl) of the supernatant were then incubated in buffer (PBS-gelatin) containing a saturating 10 nM concentration of [^{3}H]-corticosterone for 1 hr at RT. Non-specific binding was defined in parallel incubations by the addition of 2000 fold excess cold corticosterone. The samples were then incubated with DCC-G at 4°C for 10 min and centrifuged at 3000g for 15 min at 4°C to separate bound from free. Scintillation fluid was added to 900µl of supernatant in mini vials and counted. Protein content was determined by the method of Bradford [36].

Distribution of infused corticosterone in brain tissues of wild type and 11ß-HSD-1 knockout mice - Male mice aged 18 months were infused s.c. with [^{3}H]-corticosterone (6.56Ci/d, [1,2,6,7-3 H]-corticosterone, specific activity 80Ci/mmol, Amersham plc, Amersham, UK) in saline:ethanol(9:1) via Alzet mini-osmotic infusion pumps (model 2001, Alza, Palo Alto, USA). Groups of 6 wild type and knockout mice were infused for 7 days and then killed in the morning. Brains were removed, dissected and snap frozen. The content of [^{3}H]-corticosterone in each region was measured by solvent extraction with chromatographic separation of steroids. Briefly, tissues were homogenised in >9 volumes of ice-cold tris-HCl buffer (pH6.5). Homogenates were incubated for 16h at 37C with acetone:ethanol (9:1), extracts filtered and evaporated to dryness, reconstituted in buffer, washed with hexane and re-extracted with ethyl acetate. Samples were separated by TLC using UV absorption of an internal standard to localise corticosterone and radioactivity measured.

Histopathologic comparisons of retinas from aged and young mice - At autopsy, the eyes were removed and stored in paraformaldehyde at 4C. Following removal of the lens, the eyes were embedded in paraffin. Tissue blocks were sectioned (7 μm) and stained with Harris' hematoxylin and eosin. Slides were coded prior to histopathalogic evaluation at 400x magnification by an opthalmologist unaware of age or genotype.

Statistical Analysis - Data were assessed by ANOVA followed by Scheffe *post hoc* tests. Regression analysis was by the Pearson correlation matrix method. Significance was set at $p < 0.05$. Values are means±S.E.M.

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FIGURE LEGENDS

FIG. 1 (a) Impaired learning of aged wild type, but not 11ß-HSD-1$^{-/-}$ mice in the watermaze. Mice were trained for 5 consecutive days (4 trials per day) to find and escape onto a randomly located flagged hidden platform. Young 4 months old wild type (open circles), young 7 months old transgenics (open triangle), aged 18-20 months old wild type (closed circle) and aged 18-20 months old transgenics (open square). Escape latency (mean ± s.e.m.) are shown for 6 young mice per genotype and 8 aged mice per genotype, all on an isogenic 129/Ola strain background. Each point represents the average 4 trials per day. * $P<0.01$ compared to both young wild type and young 11ß-HSD-1$^{-/-}$ mice and $P<0.05$ compared to old 11ß-HSD-1$^{-/-}$ mice. (b) Failure to learn the proximally cued version of the watermaze task following hippocampal lesions in young 129/Ola mice. Hippocampal lesions were made stereotaxically by multiple injections of ibotenic acid. For sham lesions the needle was lowered through the neocortex but no drug was infused. Mice were trained for 5 consecutive days (4 trials per day) to find and escape onto a randomly located flagged hidden platform. Escape latency (mean ± s.e.m.) are shown for 5 mice per group.

FIG. 2 (a) Schematic diagram showing contribution of 11-DHC to intraneuronal corticosterone levels in wild type and 11ß-HSD-1 null mice. (b) Basal (09.00-11.00h) plasma corticosterone (cort), calculated 'free' corticosterone and 11-dehydrocorticosterone (11-DHC) and CBG levels. * $P<0.001$ compared to young wild type mice. ** $P<0.01$ compared to age matched wild type mice. Values are means ± SEM. Number of mice in brackets.

FIG. 3 Scatter plot showing correlation between morning plasma corticosterone levels and escape latency to find a flagged hidden platform on the fifth day of training for individual wild-type mice (young and old combined). The open squares represent individual young mice and the filled squares represent individual aged mice.

FIG. 4 Comparison of distribution of infused [^{3}H]-corticosterone in brain tissues of wild type and 11ß-HSD-1$^{-/-}$ mice. Aged mice (18 months) were infused s.c. with [^{3}H]-corticosterone via Alzet mini-osmotic infusion pumps for 7 days. The content of [^{3}H]-corticosterone in each brain region was measured by solvent extraction with chromatographic separation of steroids. n=6 per genotype. *, $P<0.05$ compared to wild type controls. Values are means ± s.e.m.

FIG. 5 Time spent immobile during the Porsolt swim test. A testing time of 5 min was chosen to determine the duration of immobility on each of 2 consecutive days. Immobility was defined as motionless floating in the water, only allowing small movements necessary for the animal to keep its head above the water. Mean ± s.e.m. time(s) the mice spent floating in the first session and the second session 24 h later.

Table 1.

Correlation coefficients between plasma corticosterone levels and watermaze performance in the proximally-cued platform learning (median escape latency times of the 4 trials) for each day of training in combined young and old wildtype (n= 14) and 11ß-HSD-$1^{-/-}$ mice (n=13).

	wild-type	11ß-HSD-$1^{-/-}$
Day 1	0.24 (n.s)	- 0.47 (n.s)
Day 2	0.71 **	- 0.44 (n.s)
Day 3	0.55 *	- 0.16 (n.s)
Day 4	0.69 **	- 0.58 (n.s)
Day 5	0.77 **	- 0.26 (n.s)

*P < 0.05, ** P < 0.01; n.s, non-significant

Figure 1

Dr Joyce L.W.Yau

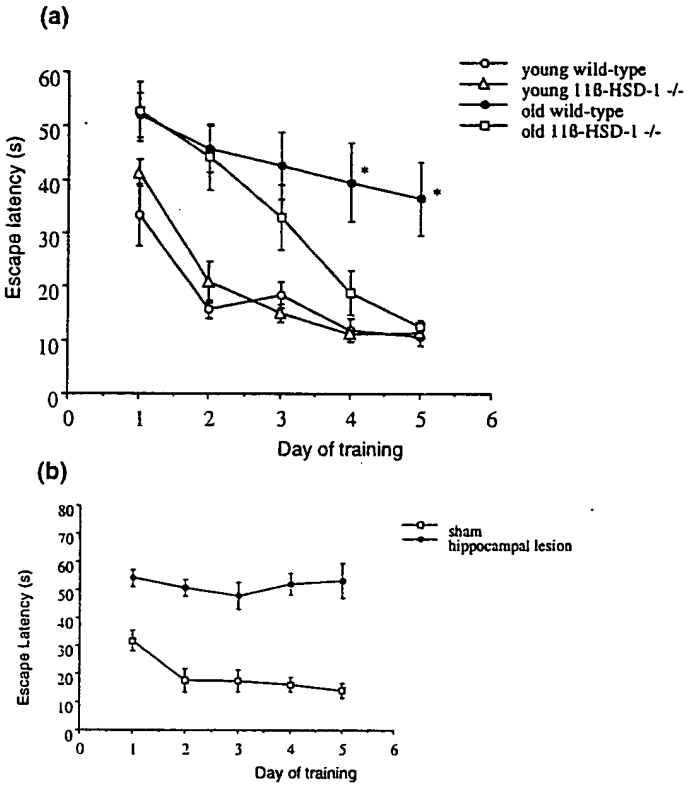

Figure 2

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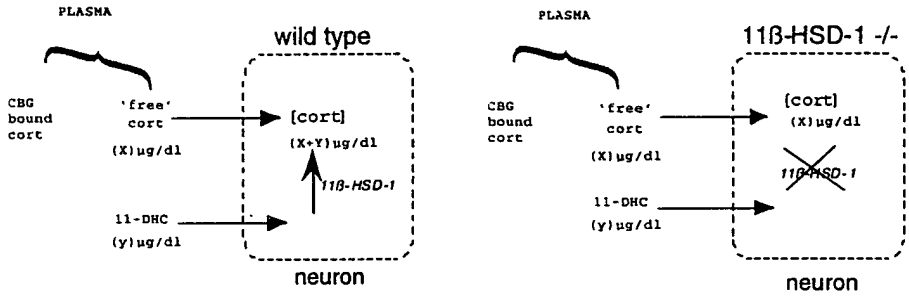
(a) Schematic diagram showing contribution of 11-DHC to intraneuronal corticosterone levels in wild type and 11ß-HSD-1 null mice.



'free' cort reflects 5% of total corticosterone (ie excludes 95% bound to CBG).

'intraneuronal' cort is the sum of 'free' corticosterone and 11-DHC in wild type mice, but only 'free' corticosterone in 11ß-HSD-1 null mice which cannot regenerate corticosterone from 11-DHC.

(b) Basal (09.00-11.00h) plasma corticosterone (cort), calculated 'free' corticosterone and 11-dehydrocorticosterone (11-DHC) and CBG levels.

	cort (µg/dl)	'free' cort (µg/dl)	11-DHC (µg/dl)	CBG (pmol/mg)
young wild-type(5)	2.5 ± 0.5	0.13 ± 0.03	0.9 ± 0.3	2.41 ± 0.28
young 11ß-HSD-1$^{-/-}$(5)	13.4 ± 2.0*	0.67 ± 0.10*	3.3 ± 0.4**	2.74 ± 0.41
old wild-type(8)	13.7 ± 1.5*	0.68 ± 0.08*	1.05 ± 0.15	2.62 ± 0.47
old 11ß-HSD-1$^{-/-}$(8)	11.2 ± 3.5*	0.56 ± 0.18*	2.6 ± 0.5**	3.30 ± 0.13

*$p<0.001$ compared to young wild-type mice. **$p < 0.05$ compared to age matched wild-type mice. Values are means±SEM. Number of mice in brackets.

Figure 3

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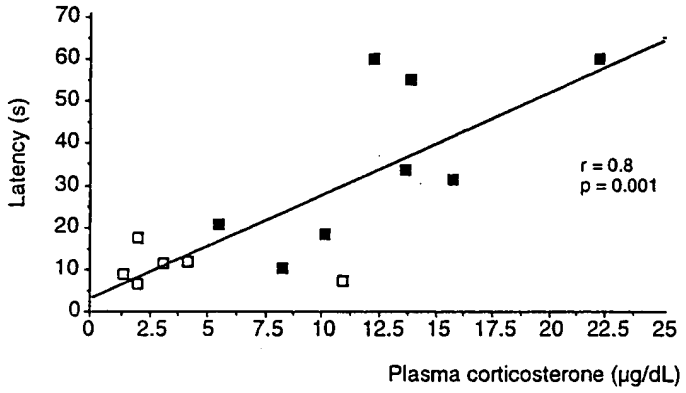

Figure 4

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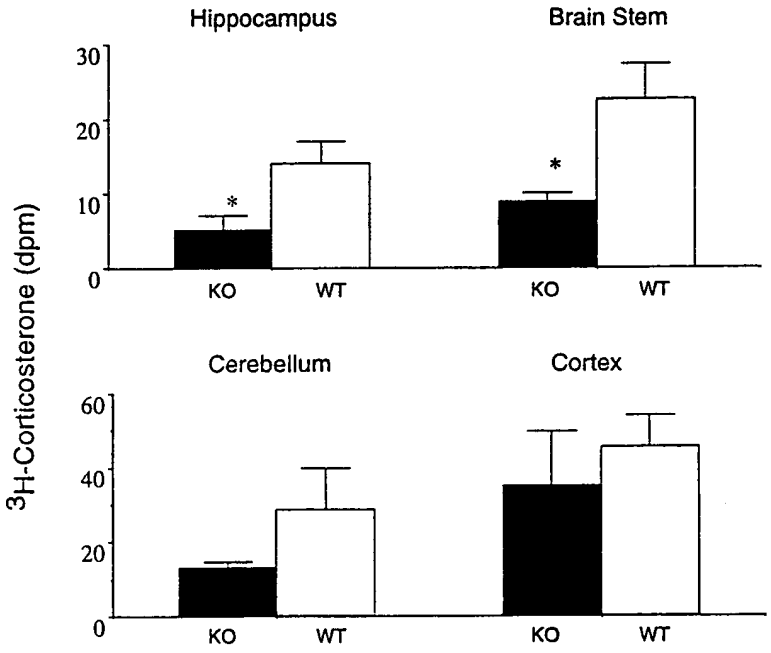

Fig 5

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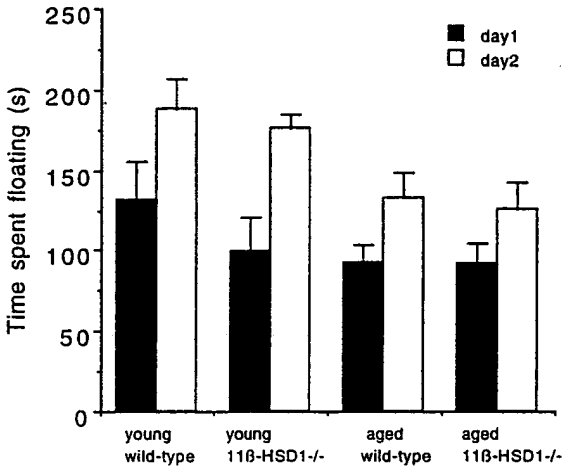

Tissue-specific dysregulation of cortisol metabolism in human obesity

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ABSTRACT Cortisol has been implicated as a pathophysiological mediator in idiopathic obesity, but circulating cortisol concentrations are not consistently elevated. The tissue-specific responses to cortisol may be influenced as much by local pre-receptor metabolism as by circulating concentrations. For example, in liver and adipose tissue cortisol is regenerated from inactive cortisone by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1). In obese Zucker rats 11β-HSD1 activity is reduced in liver but enhanced in adipose tissue. This study addressed whether the same tissue-specific disruption of cortisol metabolism occurs in human obesity. 34 men were recruited from the MONICA population study in Northern Sweden to represent a wide range of body composition and insulin sensitivity. Plasma cortisol was measured at 0830h and 1230h, after overnight low-dose dexamethasone suppression, after intravenous corticotropin releasing hormone (CRH), and after oral cortisone administration. Urinary cortisol metabolites were measured in a 24 h sample. A subcutaneous fat biopsy was obtained from 16 participants to measure cortisol metabolism *in vitro*. Higher body mass index was associated with increased total cortisol metabolite excretion ($r=0.47$, $p<0.01$), but lower plasma cortisol at 1230 h and after dexamethasone, and no difference in response to CRH. Obese men excreted a greater proportion of glucocorticoid as metabolites of cortisone rather than cortisol ($r=0.43$, $p<0.02$), and converted less cortisone to cortisol after oral administration ($r=-0.49$, $p<0.01$), suggesting impaired hepatic 11β-HSD1 activity. By contrast, *in vitro* 11β-HSD1 activity in subcutaneous adipose tissue was markedly enhanced in obese men ($r=0.66$, $p<0.01$). We conclude that in obesity, reactivation of cortisone to cortisol by 11β-HSD1 in liver is impaired, so that plasma cortisol levels tend to fall, and there may be a compensatory increase in cortisol secretion mediated by a normally functioning hypothalamic-pituitary-adrenal axis. However, changes in 11β-HSD1 are tissue-specific: strikingly enhanced reactivation of cortisone to cortisol in subcutaneous adipose tissue may exacerbate obesity; and it may be beneficial to inhibit this enzyme in adipose tissue in obese patients.

In the last 15 years it has been recognised that the effects of cortisol depend as much on tissue-specific responses as they do on circulating cortisol concentrations. Of key importance is the pre-receptor metabolism of cortisol in each tissue which dictates the balance between active and inactive steroids. Thus, in the distal nephron, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) converts cortisol to inactive cortisone; failure of this inactivation in congenital deficiency or after liquorice administration results in cortisol-dependent mineralocorticoid excess and hypertension (1). In other tissues, a different protein, 11β-HSD type 1, reactivates cortisone to active cortisol. This reactivation appears to be important to maintain adequate cortisol levels in sites where glucocorticoid receptors regulate crucial metabolic functions, eg in liver and adipose tissue (2-4).

Subtle changes in 11β-HSD activity may be important in common clinical syndromes. Given its clinical similarities to Cushing's syndrome, many investigators have considered cortisol as a pathophysiological mediator in idiopathic obesity. Although cortisol secretion rate is enhanced in obesity (5), this may be in proportion to increased lean body mass (6), and plasma cortisol levels are not consistently elevated. Metabolic clearance rate for cortisol is also increased in obesity (7). This has been explained recently by observations that reactivation of cortisone to cortisol by 11β-HSD1 in liver is impaired, and inactivation of cortisol by 5α-reductase enzymes is enhanced (8;9). These changes in metabolism predict lower plasma cortisol levels which, by negative feedback, could explain a compensatory rise in ACTH and cortisol secretion. If any increase in cortisol secretion is only to compensate for enhanced peripheral metabolism, does this mean that the tissue actions of cortisol are unaffected, ie that cortisol does not contribute to obesity and its metabolic complications?

In an animal model, the leptin-resistant obese Zucker rat, we have recently described tissue-specific changes in glucocorticoid metabolism (10). Clearance of corticosterone (the rat equivalent of cortisol) is enhanced in livers of obese animals as it is in man, due to increased 5α-reductase and impaired 11β-HSD1 activities. However, reactivation of corticosterone by 11β-HSD1 is enhanced in adipose tissue from obese rats. Moreover, obesity in these animals is glucocorticoid-dependent (11). This raises the possibility that, in the face of greater inactivation of glucocorticoid by metabolic pathways in liver, there is also enhanced reactivation of glucocorticoid in adipose tissue which might contribute to the obese phenotype. In this report we describe the same pattern of tissue-specific dysregulation of 11β-HSD1 in human obesity. These data support a new model to explain the contribution of cortisol to obesity, which provides opportunities for novel therapeutic intervention.

Materials and Methods

Participants were recruited from members of the MONICA population sample who live in the health care district of Umeå or Luleå hospitals in Northern Sweden (12). To ensure representation from a wide range of body composition, men were selected at random

from the highest and lowest quartiles of fasting plasma insulin concentrations. Thirty-five of 40 men approached agreed to participate, but one was excluded because of intermittent use of oral glucocorticoids. Characteristics of the remaining 34 are shown in Table 1. Two participants were receiving low-dose inhaled glucocorticoids (budesonide ≤400 μg/24h); two were receiving aspirin after a stroke (with no sequelae) and after mitral-valve surgery, respectively; three were receiving antihypertensive treatment (with β-blocker, ACE inhibitor, and/or calcium channel blocker). None had other clinical features of Cushing's syndrome. Diabetes mellitus and thyroid dysfunction were excluded by laboratory tests. Approval of Umeå University Hospital ethics committee and written informed consent were obtained.

Clinical measurements were made on different days separated by >24 h as follows. Days 3-5 were in random order. *Day 1)* Baseline anthropometry, blood pressure, and body composition (Akern-RJL System bioelectrical impedance instrument, EL.Dot, Fredriksvaerk, Denmark) were measured. *Day 2)* Insulin sensitivity was measured using the euglycaemic hyperinsulinaemic clamp technique, with insulin infused at 56 mU/m²/min for 110 minutes and plasma glucose maintained at 4.6±0.1 mM. *Day 3)* Plasma was obtained at 0830 h and a human CRH test (1μg/kg body weight iv) was performed at 1230 h with blood samples drawn every 15 min for 2 h; subjects did not eat until 1430 h. *Day 4)* Cortisol and its metabolites were measured in a 24 h urine sample by gas chromatography and electron impact mass spectrometry (8). *Day 5)* Conversion of cortisone to cortisol by 11β-HSD1 on first pass through the liver was measured *in vivo* after subjects took oral dexamethasone (3.5 μg/kg body weight as suspension) at 2300 h, fasted overnight, and attended at 0830 h for intravenous cannulation and oral cortisone acetate (25 mg with water)(13). Cortisol (Orion Diagnostica, PO Box 83, FIN-02101 Espoo, Finland) and corticosterone (14) were measured by radioimmunoassay on blood samples withdrawn until 1230 h. *Day 6)* Adipose 11β-HSD1 activity was measured *in vitro*. Sixteen subjects consented to return for a ~500 mg subcutaneous fat biopsy to be taken from the abdominal region under local anaesthesia. Subcutaneous fat was frozen immediately at -70C. After thawing, it was homogenised in Krebs buffer at pH 7.4 and 750 μg/ml protein was incubated at 37C with NADP 2mM and 1,2,6,7-$^3H_4$-cortisol 100 nM for 30 hours, with samples withdrawn at 3, 6, 20 and 30 hours for separation of cortisol and cortisone by HPLC with on-line liquid scintillation detection (10). 11β-HSD1 activity was measured in the dehydrogenase direction (ie cortisol to cortisone, rather than reductase cortisone to cortisol) because this is the preferred reaction when the enzyme is liberated from its intracellular environment (15); when driven with excess cofactor, this activity is proportional to total protein in whichever direction the reaction is measured (10). In these conditions, there was no evidence of conversion of cortisol to other metabolites, such as 5α-reduced cortisol.

Areas under curves of plasma cortisol after CRH or cortisone and of *in vitro* 11β-HSD1 activity were calculated by the trapezoidal rule. Logarithmic transformation was applied to obtain normal distribution of data for body mass index, waist/hip ratio, 0830 h plasma cortisol and corticosterone concentrations, urinary cortisol metabolites and their ratios, M/I insulin sensitivity index, area under curve of plasma cortisol after cortisone, and area under curve of *in vitro* 11β-HSD1 activity. Pearson correlation analyses and adjustments for potential confounding variables by partial correlation analyses were performed as indicated in the text.

Table 1. Subject characteristics and biochemical data

Tertile of BMI:	Lowest	Middle	Highest
n	11	11	12
Age (y)	46.8±8.7	49.6±8.5	51.9±12.1
Tobacco users	3	4	2
Body Mass Index (kg/m²)	22.9±1.4	26.4±0.7	31.7±4.0
% Body fat[c]	17.6±3.5	22.5±5.6	33.0±11.8
Waist/Hip ratio[c]	0.87±0.03	0.96±0.04	1.00±0.08
Insulin sensitivity (M/I)[c]	10.2±4.4	7.5±3.6	3.5±1.8
Systolic/diastolic[a] blood pressure (mmHg)	127/77 ±22/11	132/83 ±21/7	143/85 ±12/11
Urine cortisol metabolites (mg/day):			
Total[b]	11.3±5.1	12.8±5.3	15.3±3.4
5α-Tetrahydrocortisol (5α-THF)	2.1±1.0	2.1±0.8	3.1±1.1
5β-THF	2.8±0.9	3.3±1.4	2.9±1.0
Tetrahydrocortisone (THE)[b]	4.4±2.0	4.9±2.6	7.7±2.9
(5αTHF+5βTHF)/THE[a]	1.18±0.28	1.29±0.58	0.87±0.37
5αTHF/5βTHF	0.78±0.31	0.71±0.29	1.13±0.35
plasma Cortisol (nM):			
at 0830 h	285±79	358±108	282±96
at 1230 h[a]	292±49	301±100	215±78
after CRH (area under curve nM.h)	854±378	859±231	817±210
at 0830 h after dexamethasone[b]	338±117	253±145	168±106
plasma Corticosterone (nM):			
at 0830 h after dexamethasone[a]	30±11	25±12	20±10
120 min after cortisone	24±9	25±14	19±9
240 min after cortisone	18±5	17±6	13±5

Data are mean ± SD. From Pearson correlations with body mass index (BMI): [a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$. M/I=glucose infusion rate/plasma insulin concentration during euglycaemic clamp (mg glucose.kg body weight^{-1}.min^{-1}.mU/L x 100).

Results

Characteristics of participants are shown in the Table. Obese and lean subjects were well-matched for age and smoking. Generalised obesity, indicated by higher body mass index (Table 1), was associated with insulin resistance (r=-0.69), higher diastolic blood pressure (r=0.40), and higher total urinary cortisol metabolite excretion (r=0.48), but no difference in plasma cortisol at 0830 h, no difference in plasma cortisol or ACTH response to CRH, lower plasma cortisol at 1230 h (r=-0.42), and lower plasma cortisol (r=-0.52) and corticosterone (r=-0.42) at 0830 h after overnight dexamethasone suppression (Table 1 and Figure 1). Similar relationships were observed with total fat mass measured by bioimpedance, and with central obesity indicated by higher waist/hip ratio (data not shown). These associations persisted for urinary but not plasma measurements after adjustment for potential confounding factors, including age, smoking, and blood pressure. Adjustment for lean body mass did not affect these relationships, except that the correlations between body

mass index and total cortisol metabolite excretion (adjusted r=0.31, p=0.09) and 1230 h plasma cortisol (r=-0.33, p=0.07) were no longer statistically significant.

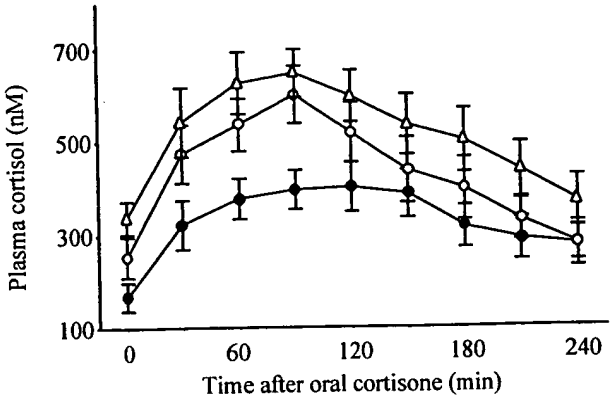

Figure 1 Plasma cortisol after overnight dexamethasone suppression and oral cortisone. Data are mean ± SE for subjects from lowest (open triangle), middle (open circle), and highest (filled circle) tertiles of body mass index. Plasma cortisol at 0830 h was lower in men with higher body mass index (r=-0.52, p<0.01). Area under curve of plasma cortisol was lower in men with higher body mass index (r=-0.49, p<0.01), independently of age, tobacco use, and blood pressure.

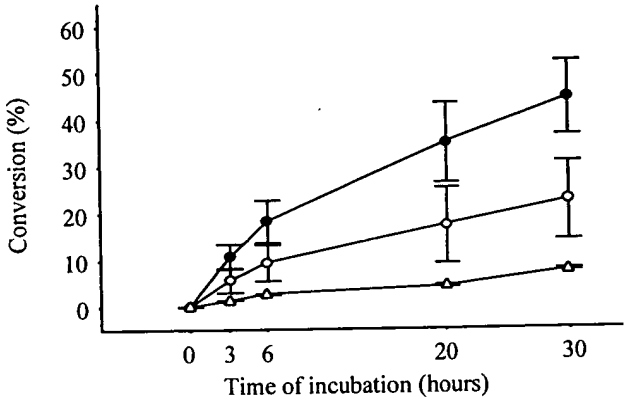

Figure 2 *In vitro* 11β-HSD1 activity in subcutaneous fat biopsy. Data are mean ± SE for % conversion of cortisol to cortisone at fixed protein concentration for subjects from lowest (open triangle, n=4), middle (open circle, n=7), and highest (filled circle, n=5) tertiles of body mass index. Areas under the curve were higher in men with higher body mass index (r=0.66, p<0.01), independently of age, tobacco use, and blood pressure.

Men with higher body mass index excreted relatively more cortisol in urine as derivatives of cortisone (tetrahydrocortisone; THE) than cortisol (Table 1), suggesting impaired reactivation of cortisone to cortisol by 11β-HSD1. Conversion of cortisone administered by mouth to cortisol in peripheral plasma was measured after overnight dexamethasone suppression, and was impaired in obese men (Figure 1). The basal 0830 h plasma cortisol before cortisone administration was lower in obese men. To assess the influence of this difference in endogenous cortisol on levels of plasma cortisol after cortisone administration we measured corticosterone, another ACTH-dependent adrenal steroid. Corticosterone was also lower after dexamethasone, but suppressed to a similar degree in obese and non-obese men after cortisone administration (Table 1). Thus, in this test, most cortisol in plasma is derived from exogenous cortisone rather than endogenous adrenal secretion. Obese men also showed trends towards higher excretion of 5α-tetrahydrocortisol (5α-THF) than 5β-THF (r=0.29).

There were no differences between participants who consented or did not consent to subcutaneous adipose biopsy (data not shown). By contrast with lower cortisol/cortisone urinary metabolite ratio and hepatic impaired conversion of cortisone to cortisol, obese men had substantially higher 11β-HSD1 activity measured *in vitro* in subcutaneous adipose tissue (Figure 2).

Insulin resistance, reflected in lower M/I values from a euglycaemic hyperinsulinaemic clamp, was associated with lower 0830 h plasma cortisol (r=0.59, p<0.001) and corticosterone (r=0.58, p<0.001) after dexamethasone and impaired conversion of cortisone to cortisol (r=0.64, p<0.001) but not with other indices of cortisol secretion or metabolism. After correction for the effect of body mass index in partial correlation analyses these relationships persisted regarding post-dexamethasone hormone levels but not for conversion of cortisone to cortisol (adjusted r=0.37, 0.43, 0.27 and p=0.03, 0.01, 0.13, respectively).

Discussion

There are several widely held explanations for increased secretion of cortisol in obesity. One is that increased cortisol secretion is appropriate to the increase in total body (lean plus fat) mass in obesity (6). A second invokes a primary neuroendocrine abnormality causing enhanced central drive to CRH, ACTH and cortisol secretion (5). A third invokes enhanced peripheral metabolism of cortisol with compensatory changes in the hypothalamic-pituitary-adrenal axis (7). In this study, we have investigated both central regulation of cortisol secretion and peripheral metabolism of cortisol. We found that increased lean body mass contributed to, but may not account entirely for, enhanced cortisol secretion. We found no evidence for enhanced central drive to cortisol secretion in these obese men, since responses to CRH were not altered and suppression with threshold doses of dexamethasone (approximately ED_{50} for suppression of plasma cortisol) was enhanced rather than impaired. More strikingly, however, our data support previous reports that peripheral metabolism of cortisol is increased due to a combination of enhanced 5α-reductase activity (8) and impaired regeneration of cortisol from cortisone by 11β-HSD1 in liver (9). One of the consequences is that levels of cortisone metabolites (THE) are elevated in obesity.

However, in animal studies dysregulation of 11β-HSD1 in obesity is tissue-specific, such that down-regulation in liver is accompanied by up-regulation in omental adipose tissue (10). Tissue-specific dysregulation of 11β-HSD1 could also explain conflicting reports of urinary cortisol/cortisone metabolite ratios in human obesity (8;9;16), since there may be a balance between impaired regeneration of cortisol in liver and enhanced regeneration in other sites. Indeed, it has been

hypothesised that increased 11β-HSD1 in omental adipose tissue is responsible for central obesity in man (4). The possibility of tissue-specific dysregulation of 11β-HSD1 is difficult to test in human studies. Measurements in omental fat have been limited to primary cell culture in non-obese subjects (4), and *in vivo* characteristics may not be retained in these cultured cells. Attempts to measure 11β-HSD1 *in vivo* in subcutaneous fat, either by arteriovenous sampling (17) or by *in vivo* microdialysis (authors' unpublished data), have produced highly variable results. In this study, we measured 11β-HSD1 activity in freshly obtained subcutaneous fat. As has been observed in extensive studies with animal tissues, 11β-HSD1 is a predominant dehydrogenase enzyme (converting cortisol to cortisone) in homogenised tissue, but the extent of this reaction reflects changes in 11β-HSD1 protein and reductase activity *in vivo* (10). The striking increase in activity in obese men predicts enhanced local reactivation of cortisone to cortisol in adipose tissue. In combination with increased supply of cortisone substrate, this predicts markedly enhanced intra-adipose cortisol levels.

We have not examined the mechanisms for tissue-specific dysregulation of peripheral cortisol metabolism, but we know that the relevant enzymes are regulated by insulin, growth hormone, and sex steroids, all of which are altered in obesity. Indeed, we found that insulin resistance was associated with impaired conversion of cortisone to cortisol in liver but not with differences in adipose 11β-HSD1 activity, but we could not confirm that this was independent of relationships with obesity. Whatever the mechanism, these observations reinforce the potential value of specific inhibitors of 11β-HSD1 to enhance insulin sensitivity (2) and limit weight gain in obesity.

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Intracellular regeneration of glucocorticoids by 11ß-HSD-1 plays a key role in regulation of the HPA axis: analysis of 11ß-HSD-1 deficient mice.

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Running Title: 11ß-HSD-1 in HPA Regulation

Abstract

11ß-hydroxysteroid dehydrogenases (11ß-HSDs) catalyse interconversion of active corticosterone and inert 11-dehydrocorticosterone, thus regulating glucocorticoid access to intracellular receptors in vivo. 11ß-HSD type 1 is a reductase, locally regenerating active glucocorticoids. To explore the role of this isozyme in the brain we examined hypothalamic-pituitary-adrenal axis (HPA) regulation in mice homozygous for a targeted disruption of the 11ß-HSD-1 gene. 11ß-HSD-1 deficient mice showed elevated plasma corticosterone and ACTH levels at the diurnal nadir, with a prolonged corticosterone peak, suggesting abnormal HPA control and enhanced circadian HPA drive. Despite elevated corticosterone levels, several hippocampal and hypothalamic glucocorticoid-sensitive mRNAs were normally expressed in 11ß-HSD-1 deficient mice implying reduced effective glucocorticoid activity within neurons. 11ß-HSD-1 deficient mice showed exaggerated ACTH and corticosterone responses to restraint stress with a delayed fall after stress, suggesting diminished glucocorticoid feedback. Indeed, 11ß-HSD-1 deficient mice were less sensitive to exogenous cortisol suppression of HPA activation. Thus 11ß-HSD-1 amplifies glucocorticoid feedback upon the HPA axis and is an important regulator of neuronal glucocorticoid exposure under both basal and stress conditions in vivo.

Keywords: 11ß-hydroxysteroid-dehydrogenase-type 1, corticosterone, 11-dehydrocorticosterone, mineralocorticoid, glucocorticoid, corticotropin-releasing factor, hypothalamic-pituitary-adrenal axis, diurnal rhythm, negative feedback, stress response.

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Introduction

Glucocorticoids regulate myriad metabolic and homeostatic processes and mediate the response to stress (1). Glucocorticoid release is stimulated by the hypothalamic-pituitary-adrenal (HPA) axis which is activated by diurnal cues and stress. These stimulate corticotropin-releasing hormone (CRH) and vasopressin release from the hypothalamic paraventricular nuclei (PVN), which induce adrenocorticotropin (ACTH) release from the anterior pituitary, stimulating adrenal glucocorticoid secretion. However, both chronic glucocorticoid excess (Cushing's syndrome, pharmacotherapy) and deficiency (Addison's disease) produce deleterious effects on many tissues and normally glucocorticoid secretion is tightly regulated by the balance of HPA axis 'forward' drive and glucocorticoid negative feedback. Glucocorticoid feedback control is mediated at the level of the PVN and anterior pituitary, as well as suprahypothalamic sites, notably the hippocampus (2). Glucocorticoids act via intracellular receptors of 2 types; glucocorticoid (GR) and mineralocorticoid (MR) receptors (3). Until recently it was believed that glucocorticoid actions upon target tissues, including the HPA axis, were determined solely by prevailing plasma steroid levels and the tissue-specific density of GR and MR. However, it has become apparent that pre-receptor metabolism by 11ß-hydroxysteroid dehydrogenases (11ß-HSDs) potently regulates glucocorticoid access to receptors in some tissues, including perhaps the brain (4).

11ß-HSD catalyses interconversion of active glucocorticoids (cortisol, corticosterone) and inert 11-keto forms (cortisone, 11-dehydrocorticosterone; 11-DHC). 11ß-HSD type 2, a potent NAD-dependent 11ß-dehydrogenase, rapidly inactivates glucocorticoids, thus allowing aldosterone-selective access to intrinsically nonselective MR in the distal nephron in the face of a large molar excess of circulating glucocorticoid (5, 6). The crucial physiological principle illuminated by the action of 11ß-HSD-2 is that corticosteroid action upon target cells is determined by enzyme action within the cells, rather than circulating steroid levels alone. Thus patients or mice lacking 11ß-HSD-2 show excess mineralocorticoid activity, despite having "normal" circulating levels of the glucocorticoids and very low levels of the mineralocorticoid aldosterone (7, 8). Hence, it is the intracellular concentration of the steroids that is important.

The 11ß-HSD-2 isozyme is not expressed in the pituitary or most regions of the adult CNS including the PVN and hippocampus (9, 10). In contrast, the brain and pituitary widely

express 11ß-HSD type 1 (11-14), an NADP(H)-dependent isozyme, initially purified from liver. 11ß-HSD-1 is bidirectional in tissue homogenates (15), but acts as a predominant 11ß-reductase in many intact cells in culture (16-19), including primary hippocampal neurons (20) and perhaps the brain (21). In principle, 11ß-reductase is anticipated to amplify glucocorticoid action in target cells, exploiting circulating inert 11-keto steroids produced largely by renal 11ß-HSD-2. 11ß-HSD-1 is highly expressed in hippocampal (12, 14) and PVN neurons (13, 22). However, any role of 11ß-HSD-1 in glucocorticoid feedback and HPA function is obscure, with studies hindered by the nonselectivity of available licorice-based inhibitors and their variable access to CNS subregions in vivo (23).

We recently produced mice homozygous for a targeted disruption of the 11ß-HSD-1 gene (24). These mice cannot regenerate active corticosterone from inert 11-dehydrocorticosterone *in vivo* confirming that 11ß-HSD-1 is the predominant or sole 11ß-reductase in the body. Despite more than adequate levels of active corticosterone in the plasma, the 11ß-HSD-1 deficient mice show reduced activation of glucocorticoid-sensitive hepatic gluconeogenic enzymes in response to stress or obesity, and consequently attenuated plasma glucose elevations to these stimuli. These data clearly illustrate the principle that intracellular activation of corticosterone from circulating inert 11-DHC plays an important role in determining effective intracellular glucocorticoid action in the hepatocyte. Indeed, the 11ß-HSD-1 null mice show elevated basal (morning) plasma levels of corticosterone, as well as adrenal hypertrophy. Whilst adrenal hypertrophy might be anticipated to reflect a requirement for increased steroid synthesis in the light of a greater, net inactivation of corticosterone in the deficient mice (with no reactivation of 11-DHC by 11ß-reductase), elevated basal levels of corticosterone would not occur if HPA axis regulation is functioning normally. In such a case there would be elevated corticosterone production over time to maintain normal plasma corticosterone levels. We have now, therefore, investigated HPA activity and glucocorticoid feedback in 11ß-HSD-1 deficient mice, to determine if circulating levels of glucocorticoids are the only signal for central actions of glucocorticoids or whether the glucocorticoid signal is modified by 11ß-HSD-1 in a tissue specific manner.

Materials and Methods

Animals

Adult 11ß-HSD-1 $^{-/-}$ male mice (MF1/129) were used for this study and compared to age-matched wild-type controls (24). Animals were housed singly prior to experiments. The light:dark cycle was kept constant with lights on from 07:00 h to 19:00 h. Animals were given standard chow and water *ad libitum* and all studies were carried out to the highest standards of humane animal care.

Materials

All chemicals were purchased from Sigma Chemical Co. (Poole, UK), unless otherwise stated. Molecular biology reagents were obtained from Promega UK.

Circadian experiments

To investigate the diurnal rhythm of glucocorticoids, animals were sacrificed by decapitation in stress-free conditions at 08:00, 12:00, 16:00, 20:00 and 24:00 h. Trunk blood was collected in EDTA (0.1% final conc.) and plasma samples stored at -80°C prior to analysis for corticosterone, 11-DHC, ACTH and corticosteroid binding globulin (CBG) levels. Brains, taken at 08:00h, were rapidly frozen on dry ice and stored at -80°C. Liver samples were removed into molybdate buffer for homogenisation and GR binding assays were performed on the extracts.

Restraint stress

For the acute restraint stress test, mice were placed in a restraint tube for 10 min and then decapitated immediately, 45 min or 90 min after the start of restraint. Trunk blood was collected, as above. At the 90 min time point, brains were taken and stored, as above.

Glucocorticoid feedback inhibition experiments

To study HPA axis feedback, basal plasma samples were obtained by tail nick, and then mice received vehicle (corn oil) or cortisol (5 mg kg $^{-1}$; 100 µl i.p., a dose chosen to cause near complete HPA axis suppression in wild-type animals) and left to recover for 2 h. Synthetic glucocorticoids, such as dexamethasone, were not used as these may poorly penetrate the CNS in rodents and are poor 11ß-HSD-1 substrates. Animals were placed in a restraint tube

for 10 min and then killed immediately or after 90 min and trunk blood collected and stored, as above.

Analysis of plasma hormones

Plasma corticosterone was measured by radioimmunoassay, as described (25), modified for microtiter plate scintillation proximity assay. Cross-reactivity with cortisol was < 8 %. ACTH was quantified by radioimmunoassay, as described (22), using commercially-available rabbit anti-ACTH antisera (IgG Corporation, Nashville, USA).

11-DHC was measured by radioimmunoassay. Plasma (20 µl) was incubated in 50 µl with rabbit anti-11-DHC antibody (1:10,000 dilution; a gift from Prof. Vecsei and Dr. Haack, Ruprecht-Karls-Universitat, Heidelberg) and [^{3}H]-11-DHC (10000 cpm) in borate buffer in a 96 well plate for 1 h at RT. Anti-rabbit SPA beads (Amersham, UK; 50 µl) were added, the plate sealed, incubated overnight at RT and counted in a Microbeta counter. The assay showed sensitivity to 11-DHC down to 2.5 nM and did not cross-react with corticosterone (in the range 0.5 – 320 nM). To reduce the concentration of free corticosterone in samples and thus any low-level cross-reactivity, non-denatured samples were used since this preserves the specific binding of corticosterone to CBG, while allowing 11-DHC to remain unbound (26). [^{3}H]-11-DHC was generated from [1,2,6,7-^{3}H]-corticosterone (Amersham, *UK*) as described (16). The efficiency of conversion of [1,2,6,7-^{3}H]-corticosterone to [^{3}H]-11-DHC was >98 %.

Plasma corticosteroid binding capacity was assessed in plasma samples, as outlined (27). Briefly, plasma samples were stripped of endogenous steroids using dextran-coated charcoal (DCC : methanol-washed charcoal (1 g) coated with dextran T-70 (0.1 g) in 100 ml molybdate buffer) and aliquots incubated with 1 pmol/100µl [1,2,6,7-^{3}H]-corticosterone (Amersham, UK) in the presence (nonspecific binding) and absence (total binding) of 2000-fold excess of unlabeled corticosterone for 1 h at 22 °C. Unbound corticosterone was removed using DCC (10 min, 4 °C) followed by centrifugation at 3000 g for 15 min at 4 °C. Bound [^{3}H]-corticosterone in the supernatant was counted. CBG in plasma was estimated from specifically-bound [^{3}H]-corticosterone. Results are presented as pmol corticosterone bound/ml plasma.

Liver cytosol GR K_d and B_{max} measurements

Liver was homogenised in 3x vol ice-cold molybdate buffer (10 mM Tris-HCl, 2 mM dithiothreitol, 1.5 mM EDTA, 0.1 M sodium molybdate, 10 % glycerol, pH 7.2) using 3x10s pulses of a Polytron homogeniser. The homogenate was centrifuged (20000g, 20 min, 4 °C) and the supernatant further centrifuged (105000g, 60 min at 4 °C) to obtain samples for protein (Bradford assay; BioRad, UK) and receptor assays. For the receptor assays, samples (50 µl) were diluted to 4 mg/ml in molybdate buffer and incubated overnight with 25 µl [1,2,4,6,7-^{3}H]-dexamethasone (final concentration 1.5 nM, 3000 cpm/assay tube; Amersham, UK) and 25 µl non-radioactive dexamethasone or corticosterone (final concentration 0.316 nM – 100 µM) at 4 °C in 96 well plates. Nonspecific binding was assessed in the presence of 200 µM cold dexamethasone. Free and bound steroids were separated using 100 µl DCC by centrifugation at 1500 g for 15 min at 4 °C. Supernatants were removed to a 96-well plate and 2 vol scintillant (Optiphase-Supermix; Wallac, Finland) added. Plates were sealed, shaken and counted. Liver cytosol GR K_d for dexamethasone (DEX; nM) and B_{max} (nmol DEX/g protein) were calculated using equilibrium binding analysis.

In situ hybridisation for GR, MR and CRH steady state mRNA expression

Coronal cryostat sections (10 µm) at the level of the hippocampus and hypothalamic PVN were mounted onto gelatin and poly-L-lysine coated slides and stored at -80°C. *In situ* hybridisation studies were performed according to (28). Tissues sections were fixed in cold 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 containing 0.02% DEPC for 10 min at room temperature, followed by 3 x 5 min in 2 x SSC. For CRH mRNA, pre-hybridisation buffer containing 50 % formamide, 0.6 M NaCl, 0.01 M Tris HCl, 1 x Denhardt's solution, 1 µM EDTA, 0.5 mg/ml denatured salmon sperm DNA and 0.125 µg/ml yeast tRNA was applied to the sections and the slides were incubated at 50 °C for 2 h in sealed boxes. Plasmids containing fragments of cDNA for rat GR (673 bp PstI-EcoRI fragment of rat cDNA), MR (513 bp EcoRI fragment of rat cDNA) and CRH (518 bp PvuII-BamHI fragment of rat cDNA) were used as templates to transcribe radiolabeled sense and antisense riboprobes using ^{35}S-UTP (Amersham, UK). cRNA probes were denatured, added to the hybridisation mixture (10 - 15 x 10^6 cpm/ml; 50 % formamide, 0.6 M NaCl, 0.01 M Tris HCl, 1 x Denhardt's solution, 1 µM EDTA, 0.1 mg/ml denatured salmon sperm DNA, 0.125 µg/ml yeast tRNA, 10 % dextran sulphate 10 min, at 75 °C), and cooled to 55°C before addition of 10 mM DTT. Hybridisation mixture (200 µl/slide) was then added to the sections and

incubated overnight (16 h at 50°C) in sealed boxes. Following hybridisation, the slides were rinsed twice in 2 x SSC for 30 min at room temperature prior to RNase A digestion (0.09 μg/ml final concentration in 0.5 M NaCl, 0.01 M Tris HCl, 0.001 M EDTA for 1 h at 37 °C). The slides were washed to a final stringency of 0.1 x SSC at 60°C. After dehydration, sections were air-dried and exposed to autoradiographic film for one week at RT. Serial dilutions of ^{35}S-UTP were made on filter paper and exposed under identical conditions to standardize signal strength. No signal was seen from ^{35}S-labeled 'sense' RNA probes of similar specific activity hybridised under identical conditions to the antisense probes (not shown). Films were quantified by computer densitometry (Research Imaging, Canada). For auto-radiographs, specific optical density measurements were obtained following subtraction of background density (obtained over white matter). Five to ten readings were taken from each region of each tissue section (3 sections per mouse). For GR, sections were dipped in photographic emulsion (Kodak NTB2, UK) and stored at 4 °C before development and counterstaining with pyronin (1%,w/v). Grain counting was performed using an MCID system (Research Imaging, Canada).

Statistics

For diurnal rhythms and stress experiments, groups were compared by genotype and time using 2-way ANOVA. Where significant differences and a genotype:time interaction were noted, one-way ANOVA with *post-hoc* Scheffe F-tests were performed for within group comparisons, and t-tests were used to show significant differences between groups. For *in situ* hybridization histochemistry data, groups were compared by independent two-tailed Student's t-tests. Some hormonal data were not normally distributed and were log-transformed before analysis, or, when this failed to normalize the distribution (eg basal ACTH), data were assessed by the non-parametric Mann-Witney test. Values are means ± sem (n) and $p<0.05$ was taken as significant.

Results

11-HSD-1 deficient mice have altered basal HPA parameters and diurnal rhythms

Wild-type mice showed a clear diurnal rhythm of plasma corticosterone, with a nadir of ~25 nM at 08:00 h and a peak of ~150 nM at 20:00 h (Figure 1A). Plasma corticosterone levels in 11ß-HSD-1 $^{-/-}$ animals were elevated at 08.00h, compared with wild type controls. The 11ß-HSD-1 $^{-/-}$ mice also showed a diurnal rhythm of corticosterone, with a nadir at 08:00 h, but

had an earlier rise at 12:00 h, resulting in a more extended corticosterone peak. The 11ß-HSD-1 deficient mice showed significantly greater corticosterone secretion over the 24 h period (genotype:time interaction, $F(4,66)=3.41$, $p=0.0135$).

Plasma 11-DHC levels in wild type mice also showed a significant diurnal variation, approximately in parallel to plasma corticosterone, with a nadir at 08:00 h and a peak at 20:00 h (Figure 1B). 11-DHC levels in 11ß-HSD-1 deficient mice were significantly elevated compared to wild-type animals over the 24 h period (genotype:time interaction, $F(4,76)=4.678$ $p=0.002$). 11ß-HSD-$1^{-/-}$ animals maintained the diurnal rhythm of 11-DHC, with levels significantly elevated above morning basal levels by 12:00 h and an earlier peak than wild-type animals.

11ß-HSD-1 deficient mice showed a striking reduction in the amplitude of the diurnal ACTH rhythm compared to the wildtype mice. Although peak levels were similar, nadir plasma ACTH levels were higher in 11ß-HSD-$1^{-/-}$ mice ($p=0.02$ Mann-Witney test; Figure 1C).

Plasma CBG levels (Table 1) and hepatic GR binding characteristics (Table 1) were similar in wild-type and 11ß-HSD-$1^{-/-}$ animals, demonstrating that alterations in these parameters cannot underlie the changes in basal HPA activity in the 11ß-HSD-$1^{-/-}$ mice. GR and MR mRNA levels in the hippocampus were also identical between the genotypes (Table 2). Whilst CRH mRNA expression in the hypothalamic PVN was similar in wild-type and 11ß-HSD$1^{-/-}$ mice, GR mRNA levels in the PVN were significantly reduced in 11ß-HSD-$1^{-/-}$ mice ($p<0.01$; Table 2).

11-HSD- 1 deficient mice show elevated responses to acute restraint stress

Following 10 min restraint stress, both wild-type and 11ß-HSD$1^{-/-}$ mice showed significant elevation of plasma corticosterone, but the peak corticosterone response of the 11ß-HSD-$1^{-/-}$ mice was significantly greater than in wild-type (Figure 2A) and overall the 11ß-HSD-$1^{-/-}$ mice had a greater corticosterone response to stress compared to wild-type (genotype:time interaction, $F(3,54)=5.48$ $p=0.0023$). Whilst the initial fall in plasma corticosterone levels was rapid in both genotypes, 90 min after stress, plasma corticosterone levels were significantly higher in 11ß-HSD-$1^{-/-}$ mice than wild-type ($p<0.05$).

In wild-type mice restraint stress produced a gradual increase in plasma 11-DHC over the sampling period, the increase only reaching significance at 90 min (Figure 2B). In marked contrast, 11-DHC levels in 11ß-HSD-1 deficient mice increased dramatically, peaking 10 min following restraint stress and falling thereafter. The peak levels achieved were significantly increased in the mutant mice above those seen in wild-type mice (genotype:time interaction, $F(3,59)=4.467$, $p=0.0068$).

Both 11ß-HSD-1 deficient and wild type mice showed an immediate ACTH response to restraint stress. However, the overall ACTH response to stress was significantly greater in the 11ß-HSD-$1^{-/-}$ mice (genotype:time interaction, $F(3,29)=6.059$, $p=0025$). Moreover, ACTH levels remained elevated above basal values for a longer period (90 min) in the 11ß-HSD-1 deficient animals (Figure 2C).

Reduced effect of exogenous glucocorticoids on HPA axis activity in 11-HSD-1 deficient mice

Wild type mice were pre-treated 2 h prior to stress with vehicle or cortisol in a dose chosen (on the basis of pilot studies) to cause near full inhibition of the subsequent corticosterone response to restraint stress (cortisol-treated corticosterone peak 66 ± 17 nM, n=5). In age and weight-matched 11ß-HSD-$1^{-/-}$ mice, this dose of cortisol was significantly less effective in suppressing the corticosterone rise 10 min post-stress (cortisol 114 ± 8 nM, n=4). Thus at 10 min, immediately on cessation of stress, wild-type animals pretreated with cortisol, had significantly lower corticosterone levels compared to 11ß-HSD-$1^{-/-}$ mice pretreated with cortisol (Figure 3).

Discussion

Here we show that deficiency of 11ß-HSD-1 alters HPA function, both basally and in response to stress. 11ß-HSD-1 has been shown to be the only enzyme to convert inactive 11-deoxycorticosterone (11-DHC) to active corticosterone in the mouse (24). Therefore, although normal conversion of corticosterone to 11-DHC occurs in the 11ß-HSD-1-/- mice, no regeneration of corticosterone within tissues occurs, due to the lack of 11-reductase activity. This may be predicted to result in lower glucocorticoid levels and hence a compensatory increase in basal corticosterone secretion would ensue to maintain the normal corticosterone

levels. The maintenance of basal corticosterone levels appears to be critical for normal functioning of the HPA axis and its response to various stressors (29). Indeed 11ß-HSD-1 deficient mice show adrenocortical hyperplasia and increased adrenal sensitivity to ACTH *in vitro* (24). However, basal plasma corticosterone levels should be unaltered, providing HPA sensitivity to glucocorticoids is unchanged. Instead, 11ß-HSD-1 deficient mice have clear basal glucocorticoid hypersecretion alongside increased basal ACTH levels. These data imply increased basal HPA activity which might be due to either increased drive and/or attenuated feedback control.

Several lines of evidence demonstrate that 11ß-HSD-1 deficient mice have reduced sensitivity to glucocorticoid negative feedback upon the HPA axis. First, despite elevated circulating glucocorticoid levels, there is no down-regulation of several key HPA-associated glucocorticoid target genes and their products, including GR and MR mRNAs in the hippocampus, CRH mRNA in the PVN and plasma ACTH levels. The latter is particularly unexpected, given the increased adrenal sensitivity to ACTH in these mice (24). Second, after stress, the rate at which plasma corticosterone levels return to basal (post-peak) is dependent on the ability of glucocorticoids to terminate the central activation of HPA activity at various sites (pituitary, hypothalamus and higher centres of the brain) by negative feedback. Despite elevated peak glucocorticoid levels in response to stress and the more efficient clearance of corticosterone, in the absence of 11ß-reductase regeneration from 11-DHC, post peak (60-90 min) plasma ACTH and corticosterone levels are significantly higher in 11ß-HSD-1 deficient mice than wild type controls. Hence elevated post-peak plasma corticosterone and ACTH implies insensitivity to feedback control. Third; directly to address this, mice were given cortisol in a dose which greatly attenuated the HPA response to a subsequent stress in wild-type mice. 11ß-HSD-1 deficient mice similarly pretreated with cortisol had a significantly greater corticosterone response to stress, despite increased metabolic clearance of cortisol (no regeneration by hepatic 11ß-HSD-1 after metabolism to cortisone by renal 11ß-HSD-2 which is unaltered in the 11ß-HSD-1 deficient mice (24)), confirming a lack of sensitivity to glucocorticoid feedback in these animals.

Insensitivity to negative feedback regulation by glucocorticoid hormones could be obtained in several ways; decreased free levels of circulating glucocorticoids by elevation of plasma CBG levels, by decreased glucocorticoid levels in relevant tissues due to altered 11ß-reductase

activity, or changes in tissue GR affinity or numbers at sites of negative feedback regulation (particularly the hippocampus and hypothalamus). However, no changes were observed in CBG levels in 11ß-HSD-1 deficient mice. Indeed, CBG is a hepatic glucocorticoid-inhibited transcript (30). The maintenance of CBG levels in 11ß-HSD-1 deficient mice further supports the notion of reduced effective glucocorticoid action in cells despite plasma glucocorticoid excess. The insensitivity to feedback cannot readily be ascribed to alterations in GR affinity for glucocorticoids which was unaltered (at least in liver) or in GR or MR mRNA levels in the hippocampus. Hence, the implication is that lack of 11ß-reductase in feedback sites such as hippocampus, pituitary and perhaps PVN leads to attenuated glucocorticoid regeneration within these cells and therefore a reduced effective intracellular glucocorticoid signal in vivo.

However, there is a tissue-specific heterogeneity of glucocorticoid effects in the 11ß-HSD-1 deficient mice. Notably, the animals show reduced GR mRNA in the PVN. This contrasts with maintained GR in the hippocampus and indeed maintained CRH mRNA in the same region of the PVN. These findings suggest that there is a differential sensitivity to corticosterone in different brain areas. Activity of 11ß-HSD-1 is lower in the hypothalamus than the hippocampus and pituitary (12, 14), which is in accord with these findings and supports the notion that the enzyme provides tissue-specific glucocorticoid modulation. Lower GR in PVN might attenuate feedback sensitivity too and may underpin the maintenance of CRH transcripts in the face of hypercorticosteronaemia. It is likely that a combination of two processes, reduced GR expression in PVN and loss of 11ß-reductase with consequent decrease in ligand availability to GR, underlies the negative feedback insensitivity in 11ß-HSD-1 deficient mice. The importance of GR in negative feedback sensitivity of the HPA axis has been demonstrated in mice which lack GR throughout the CNS (31). These animals show elevated CRH and corticosterone, but reduced ACTH levels, suggesting the expected dominant role for the receptor, but illustrating the analogous though less potent effect of 11ß-HSD-1. However, *reduction* of GR by antisense expression (32) has less effect upon basal corticosterone and ACTH (33) than loss of 11ß-HSD-1, suggesting that absence of enzyme activity in 11ß-HSD-1 deficient mice is the more critical factor contributing to the HPA abnormalities than the modest reduction in GR seen in the PVN in the 11ß-HSD-1 deficient mice.

Interestingly, circulating levels of 11-DHC (which are little bound to plasma proteins) in mice are 2-7 nM and thus similar to, if not greater than, basal 'free' corticosterone levels (~1nM), assuming ~95% of circulating corticosterone is usually bound to CBG. Cortisone levels in humans are even higher at around 50nM (34). Moreover, ~50% of 11-DHC is converted to active corticosterone on a single pass through the intact rat liver (35). It is therefore feasible that 11ß-reductase activation of 11-DHC contributes an appreciable proportion of intracellular active glucocorticoid. The progressive accumulation of 11-DHC following stress in wild type mice is presumably a consequence of increased corticosterone substrate for renal 11ß-HSD-2 conversion to 11-DHC which is then back-converted to corticosterone by 11ß-HSD-1 in liver and elsewhere, fueling further renal conversion to 11-DHC. The rising 11-DHC levels presumably also represent an increasing substrate for reduction in feedback sites, amplifying the glucocorticoid effect.

What of forward HPA drive? The 11ß-HSD-1 deficient mice show maintained circadian rhythmicity and good HPA responses to stress suggesting stimulatory pathways are intact. The exaggerated early stress responses of corticosterone are consistent with increased adrenal sensitivity to ACTH with 'paradoxically' maintained ACTH release, presumably due to the attenuated central sensitivity to the elevated basal glucocorticoid levels. Similar central glucocorticoid insensitivity (but not adrenal hypersensitivity) is seen in transgenic mice with antisense reduced GR expression (33, 36). However there is a diurnal early peak of HPA activation in the 11ß-HSD-1 deficient mice suggesting more fundamental alterations in rhythm generation, perhaps at the suprachiasmatic nucleus which expresses some 11ß-HSD-1 (12) and is sensitive to glucocorticoid modulation. Future studies will address the nature of this finding and the possible relevance of 11ß-HSD-1 in the human CNS and its disorders. Overall, the data suggest a novel and important role for 11ß-reductase in regulation of the HPA axis. The importance of this enzyme in other CNS systems remains to be determined. This is of particular interest in the hippocampus and cerebellum where 11ß-HSD-1 expression is highest and where glucocorticoids have important effects upon electrophysiological parameters, behavior and cognition as well as neuronal development, structure and survival (3, 37, 38).

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Table 1: Plasma corticosteroid binding globulin (CBG) levels and liver glucocorticoid receptor (GR) kD for dexamethasone (DEX, nM) and Bmax (nmol DEX/g protein) from wild-type (+/+) and 11ß-HSD-1 null mice (-/-). Values are mean±SEM, n=9.

	+/+	-/-
Plasma CBG (pmol corticosterone/ml)	217±23	174±15
Liver GR binding		
kD (nM)	1.9±0.6	2.0± 0.5
Bmax (nmol/g protein)	0.14± 0.02	0.17±0.03

Table 2: Basal (nadir) steady state mRNA levels of glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and corticotropin-releasing hormone (CRH) in wild-type (+/+) and 11ß-HSD-1 null mice (-/-) in the hypothalamic paraventricular nucleus (PVN), and dentate gyrus (DG), CA1 and CA3 regions of the hippocampus. In situ hybridization studies assessed by grain counting over individual neurons (GR) or optical density measurements over autoradiographically identified areas (MR, CRH). Data are means ± SEM (n); ** $p<0.01$ compared to wild-type value; n.d. = not determined

	GR (grains/cell)		**MR** (OD; as % +/+ DG)		**CRH** (OD; as % +/+)	
	+/+	-/-	+/+	-/-	+/+	-/-
PVN	46±6 (7)	25±3 (8) **	n.d.	n.d.	100±6 (10)	89±5 (12)
DG	37±6 (8)	31±4 (8)	100±11 (9)	83±16 (9)	n.d.	n.d.
CA1	38±9 (8)	32±4 (8)	96±10 (10)	107±19 (9)	n.d.	n.d.
CA3	11±3 (8)	13±2 (8)	180±23 (10)	196±43 (10)	n.d.	n.d..

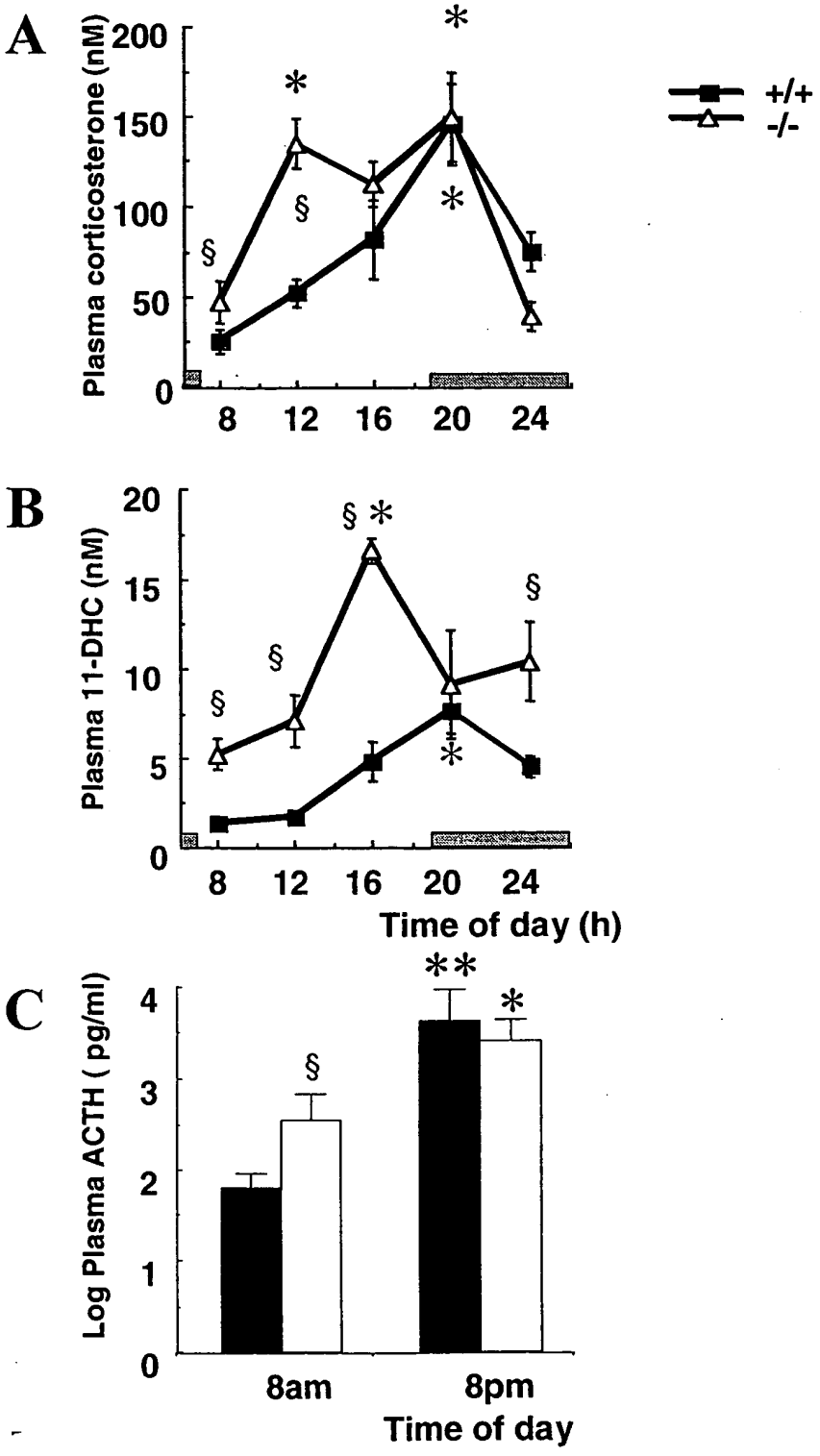
Figure Legends

Figure 1: Plasma levels of **A**) corticosterone (nM), **B**) 11-dehydrocorticosterone (11-DHC; nM) and **C**) Log plasma levels of adrenocorticotropin (ACTH; pg/ml) from wild-type (+/+ filled squares) and 11ß-HSD-1 null mice (-/- open triangles) secreted throughout the day (lights on 07:00 h to 19:00 h). Values are mean±SEM, n=6-9 mice for each time point. *$p<0.05$, **$p<0.01$ compared to 08:00 h time point, § $p<0.05$ compared to wild-type at that time point.

Figure 2: Plasma levels of A) corticosterone (nM), B) 11-dehydocorticosterone (11-DHC; nM) and C) Log plasma levels of adrenocorticotropin (ACTH; pg/ml) secreted over 90 min from wild-type (+/+ filled squares) and 11ß-HSD-1 null mice (-/- open triangles) in response to 10 min restraint stress (hatched bar) given at 09:00 h. Values are mean±SEM, n=7-10 mice for each time point. * $p<0.05$ compared to wild-type at that time point.

Figure 3: Plasma levels of corticosterone (nM) secreted over 90 min from wild-type (+/+ filled column) and 11ß-HSD-1 null mice (-/- open column) in response to 10 min restraint stress given at 11:00 h, 2 h after pre-treatment with cortisol (5 mg/kg i.p.). Values are mean±SEM, (n). * $p<0.05$ compared to wild-type.

Figure 1


A
Plasma corticosterone (nM)
200
150
100
50
0
8
12
16
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B
Plasma 11-DHC (nM)
20
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Time of day (h)
C
Log Plasma ACTH (pg/ml)
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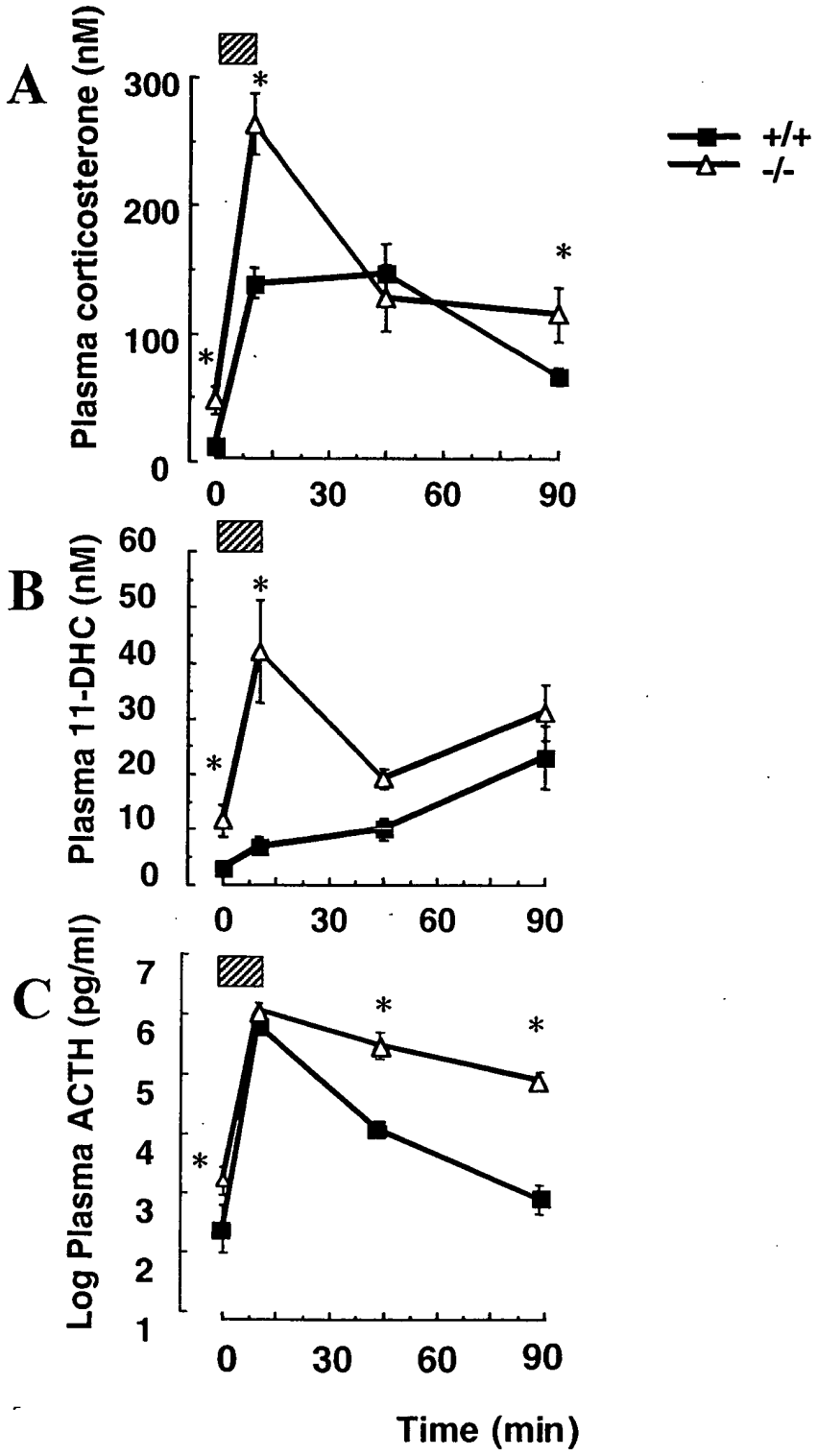
8am
8pm
Time of day

Figure 2


A
Plasma corticosterone (nM)
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200
100
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30
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90
+/+
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B
Plasma 11-DHC (nM)
60
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40
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C
Log Plasma ACTH (pg/ml)
7
6
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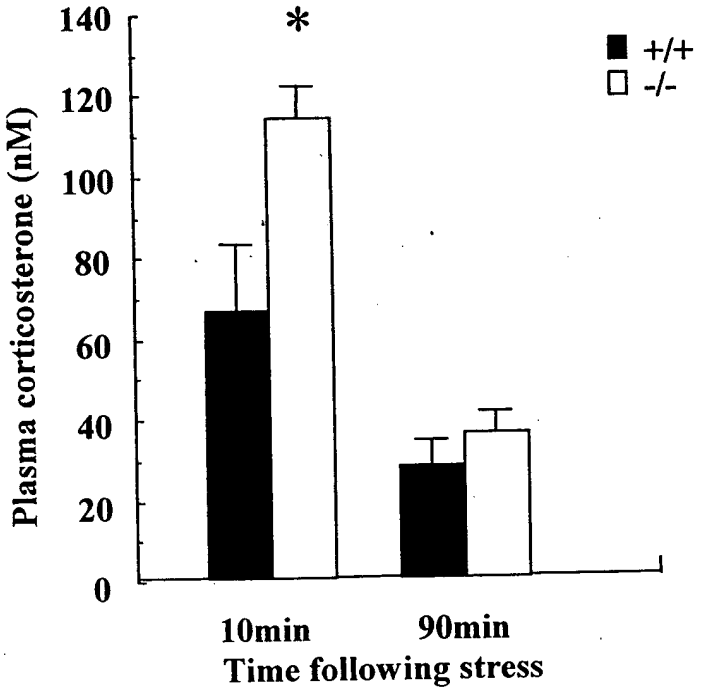
1
Time (min)

Figure 3


140
120
100
80
60
40
20
0
*
+/+
-/-
Plasma corticosterone (nM)
10min
90min
Time following stress

11β–Hydroxysteroid dehydrogenase Type 1 Null Mice have an Atheroprotective Lipid Profile.

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Running Title: Atheroprotective Lipid Profile in 11βHSD-1 Null Mice.

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Summary

Strong evidence suggests 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD-1) acts mainly as a tissue-specific 11β-reductase, thus amplifying intracellular glucocorticoid action by inter converting inert 11-dehydrocorticosterone to active corticosterone, particularly in liver. 11βHSD-1 null mice (11βHSD-$1^{-/-}$) mice resist the hyperglycaemia resulting from stress and chronic high-fat feeding. Here we investigated aspects of lipid metabolism, hepatic insulin sensitivity and cardiovascular function determined by liver-specific changes in gene expression in 11βHSD-$1^{-/-}$ mice. Ad lib fed 11βHSD-$1^{-/-}$ mice have lower triglyceride and elevated HDL cholesterol levels compared to wild-type. Whilst liver transcript levels of proteins in lipogenic pathways are similar to wild type, those encoding enzymes of fatty acid oxidation are increased in 11βHSD-$1^{-/-}$ mice. Hepatic expression of PPARα, a key glucocorticoid-sensitive transcription factor driving lipid metabolism, is also elevated in fed 11βHSD-$1^{-/-}$ mice. HDL apolipoprotein AI transcript and serum levels are elevated whereas serum apolipoproteins AII, CIII and liver Aα-fibrinogen transcript levels are reduced. Despite an abolished PPARα induction, fasting glucose levels and induction of the fatty acid oxidation pathway in 11βHSD-$1^{-/-}$ mice is similar to wild type. Further, re-fed 11βHSD-$1^{-/-}$ mice have increased hepatic insulin sensitivity, with exaggerated repression or induction of insulin-sensitive transcripts in the oxidative and lipogenic pathways, respectively. Finally, intraperitoneal glucose tolerance tests show 11βHSD-$1^{-/-}$ mice have improved glycaemic control. These data provide a molecular basis for the contention that functional loss of 11βHSD-1 promotes a favourable, potentially atheroprotective and insulin sensitised phenotype in 11βHSD-$1^{-/-}$ mice.

Introduction

Glucocorticoids potently regulate glucose and lipid homeostasis, acting largely via intracellular glucocorticoid receptors in the liver, adipose tissue and muscle. Glucocorticoid actions are mainly, but not exclusively, antagonistic to the effects of insulin. Excess glucocorticoids, epitomised by Cushing's syndrome in humans, leads to insulin resistance/type 2 diabetes, dyslipidaemia and a redistribution of adipose tissue to the visceral depots associated with increased cardiovascular risk (1). It has been suggested that more subtle abnormalities of glucocorticoid action within cells may occur in the "metabolic" or insulin resistance syndrome of co-associated cardiovascular risk factors (2-4). Recently a novel and important level of control of glucocorticoid action has become apparent, pre-receptor metabolism by 11ß-hydroxysteroid dehydrogenases (11ß-HSDs). 11ß-HSDs catalyse the interconversion of active physiological 11-hydroxy glucocorticoids (cortisol in most mammals, corticosterone in rats and mice) and their inert 11-keto forms (cortisone, 11-dehydrocorticosterone). There are two isozymes of 11ß-HSD, the products of distinct genes (5, 6). 11ß-HSD type 2 is a high affinity dehydrogenase that rapidly inactivates corticosterone in kidney and colon, thus excluding glucocorticoids from otherwise non-selective mineralocorticoid receptors *in vivo* (7, 8). However, white adipose tissue solely expresses 11ß-HSD type 1 (9), as does the liver where the enzyme is particularly abundant (10, 11).

11ß-HSD-1 is a predominant reductase in most intact cells, including hepatocytes (12), adipocytes (13), neurons (14), and in the isolated liver *ex vivo* (15). This reaction direction regenerates active glucocorticoids within cells from free circulating inert 11-ketosteroids. Mice homozygous for targeted disruption of the 11ßHSD-1 gene are viable, fertile and have normal longevity (16). However, 11ßHSD-1 null mice cannot regenerate corticosterone from inert 11-dehydrocorticosterone, indicating this isozyme is the unique 11ß-reductase. Strikingly, the null animals exhibit attenuated gluconeogenic responses upon stress and resists the hyperglycaemia induced by chronic high fat feeding (16). This occurs despite modestly elevated plasma levels of corticosterone. The results suggest that 11ßHSD-1-reductase activity is an important amplifier of intrahepatic glucocorticoid action *in vivo*. Intriguingly, tissue-specific alterations in 11βHSD-1 activity have been implicated in the development of obesity and insulin resistance in obese Zucker rats (4) and in humans (2).

In the Metabolic Syndrome, dyslipidaemia is characterised by hypertriglyceridaemia and an aberrant lipoprotein and cholesterol profile with elevated VLDL[1], but reduced 'cardioprotective' HDL cholesterol (17). The plasma lipid profile is largely determined by gene expression in the liver. Furthermore, expression and activity of many liver proteins involved in lipid metabolism, synthesis, packaging and export are glucocorticoid-sensitive. However, the precise role of glucocorticoids in the pathogenesis of hepatic lipid metabolism is unclear, with overall effects apparently dependent upon steroid concentrations, the levels of other hormones, particularly insulin, and on diet. Indeed, many studies have used short-term treatments and/or non-physiological levels of glucocorticoids, making any extrapolations of the subtle effects of altered intracellular glucocorticoid metabolism difficult. Moreover, glucocorticoids also have important indirect effects, regulating other key transcription factors controlling lipid metabolism, notably inducing the peroxisome proliferator-activated receptor-α (PPARα) (18, 19). PPARα drives the oxidative adaptation to fasting (20, 21) and serves as the molecular target of hypolipidaemic fibrate drugs (22, 23).

Here we investigate the hypothesis that 11ß-HSD-1$^{-/-}$ mice have an altered cardiovascular risk profile due to liver-dependent changes in lipid metabolism and insulin sensitivity. To address these issues we have analysed circulating lipids and lipoproteins and assessed expression of hepatic genes

involved in lipid metabolism and transport, as well as fibrinogen, another glucocorticoid-sensitive hepatic transcript associated with cardiovascular risk.

[1]The abbreviations are: VLDL, very low density lipoprotein; HDL, high density lipoprotein; apo, apolipoprotein; SREBP-1c, sterol regulatory element-binding protein-1c; FPLC, fast protein liquid chromatography; PEPCK, phosphoenolpyruvate carboxykinase, G-6-P, glucose-6 phosphatase; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal axis; CBG, corticosterone binding globulin.

Experimental Procedures

Animals – Male wild type MF1 11βHSD-1$^{-/-}$ mice and their controls, bred as previously described (16), were housed in standard conditions on a 12h light: 12h dark cycle (lights on 7am). For experiments, animals were housed singly and allowed to acclimatise for at least two days. Animals were allocated at random (n=6 per group) to receive either ad lib access to chow, a 24 h fast, a 24 h fast with a 4 h re-feed or a 24 h fast with a 24 h re-feed. All fasting commenced at 8am. Animals were killed by decapitation in a separate room from their housing within 1 min of their cage being disturbed.

Plasma Parameters –Trunk blood was collected rapidly, plasma separated and samples kept on ice until measurement of triglyceride, free fatty acids, total cholesterol and HDL cholesterol. Triglyceride was measured using a lipase based colourometric TG kit (Roche, Mannheim, GmbH). Total and HDL cholesterol were measured using the CHOL and HDL C-Plus kits (Roche). Glucose was measured with a Glucose HK kit (Sigma, Poole, UK). Plasma insulin was measured using an ELISA performed according to manufacturers instructions (Crystalchem, Chicago, USA). Corticosterone levels were determined by radioimmunoassay, as described (24) *Catherine Feivet and Bart Staels* -Serum was also obtained and analysed for true triglyceride (glycerol-free peak by FPLC separation) and cholesterol profiles by FPLC followed by enzymatic methods as previously described (23). ApoAI, apoAII, apoCII, apoB and apoE were measured by nephelometry using specific antibodies on representative samples.

RNA Extraction and Analysis – Tissues were snap-frozen in liquid nitrogen and homogenised in Trizol (Gibco BRL, Paisley, UK). Total RNA was purified by adding a binding matrix (Rnaid Plus kit, BIO 101, Anachem, UK) and eluted from the matrix in diethylpyrocarbonate (Sigma) pre-treated water containing 400units per ml RNAsin (Promega, Southampton, UK) and 10mM DTT. RNA (5-20μg) was resolved on a 1% MOPS formaldehyde gel and blotted according to standard northern blot procedure in 20x SSC onto Hybond N^+ membranes (Amersham, Little Chalfont, UK). Probes were labelled with ^{32P}d-CTP using a random primed labelling kit (Roche), purified through Nick Columns (Pharmacia-Amersham, Little Chalfont, UK) and hybridised overnight in high SDS (6%) phosphate buffer (0.2M NaH_2PO_4, 0.6M Na_2HPO_4, 5mM EDTA) containing 0.5mg/ml denatured salmon testes DNA (Sigma) at 65°C. Blots were washed at 65°C to a maximum stringency of 0.5xSSC, 0.1%SDS, exposed to phosphor imager film (FLA2000, Fujifilm, London, UK) and analysed by quantitative phosphor imager software (Aida, Raytek Scientific, Sheffield, UK). Blots were also exposed to film (biomax-MR, Kodak, UK). The probes used for this study are listed in table I and were generated from primers designed to sequences in Genbank. All probe identities were confirmed by sequencing using the Thermosequenase kit (USB, Cleveland, USA) on standard 8% acrylamide sequencing gels.

Intraperitoneal Glucose Tolerance Test – In a separate experiment, transgenic and wild type mice were fasted overnight and then injected intraperitoneally with 2mg/g D-glucose (25% stock solution in phosphate buffered saline). Blood samples were taken by tail venesection into EDTA-micro tubes (Sarstedt, Leicester, UK) at zero (before injection and within 1 minute of disturbing the cage) and at 5, 15 30 60 and 120 minute intervals after the glucose load.

11β-HSD-1 Enzyme Activity – Liver samples were homogenised as described (12). The reaction included 0.1mg/ml protein, 25 nM tritiated corticosterone and an excess (2μM) of the 11βHSD-1 specific co-factor NADP (under *in vitro* conditions in homogenised tissues, 11ß-HSD1 is bi-directional, with assay of dehydrogenation more stable). The assay was in the linear range of protein concentration and product formation. After a 10 min incubation, steroids were extracted with ethylacetate. Steroids were separated by thin layer chromatography (TLC), identified by

migration in comparison to unlabelled corticosterone and 11-dehydrocorticosterone standards and quantified with a phosphorimager tritium screen (Fujifilm). TLC results were validated by HPLC analysis of a representative group of samples from each experimental group..

Oil Red O Staining for Lipid – Frozen livers were sectioned (20μm) and stained with Oil red O (Sigma) to identify neutral lipid, cholesterol and fatty acids (red colour). Nuclei were counterstained with (blue) haematoxylin (Sigma). Rinses were as follows: water, 60% isopropanol, 0.1% w/v oil red O in isopropanol 10 min, 60% isopropanol, 3x water, mayers haematoxylin 90 s, 1% ammonium, water. Sections from livers in each experimental group were processed simultaneously. Finally, slides were covered in aqueous mount, coverslipped and viewed under a light microscope at equal light intensity.

Results

11βHSD-1$^{-/-}$ mice have lower plasma triglyceride and higher HDL cholesterol – Plasma triglycerides were lower in ad lib fed 11βHSD-1 null mice (Fig.1A). A representative FPLC profile of *ad lib* 'true' triglycerides (Fig. 1B) indicated that glycerol interference does not account for the differences between genotype. Triglycerides clearly fall upon fasting in both genotypes. Two way ANOVA indicated that the reduction in triglycerides in 11βHSD-1$^{-/-}$ mice upon fasting was significantly smaller in magnitude compared to wild type (Fig 1A). However, whilst wild-type triglyceride levels returned to *ad lib* fed values by 24 hours of re-feeding, 11βHSD-1$^{-/-}$ triglyceride values returned to ad lib values by 4 hours and exhibited an overshoot to levels significantly higher than the *ad lib* fed group at 24 hours. Total and HDL cholesterol did not vary significantly with dietary manipulation (Fig. 2A and 2B). However, there was a highly significant effect of genotype, with 11βHSD-1$^{-/-}$ mice having higher HDL cholesterol levels (~130% of wild type; Fig. 2B). Plasma glucose levels were similar in both genotypes in the fed state (wild type 6.24±0.04 versus null 5.8±0.5 mmol/L), with a trend towards lower fasting glucose in 11βHSD-1$^{-/-}$ mice (wild type 4.04±0.3 versus null 3.4±0.1 mmol/L), as previously observed (Kotelevtsev et al 1997). Four hours re-fed glucose levels were similar (wild type 5.37±0.45 versus null 5.51±0.29 mmol/L), however, there was a small but significant decrease in 11βHSD-1 null glucose levels at 24 hours re-fed after a fast (wild type 5.51±0.45 versus null 4.64±0.15 mmol/L, $p<0.05$). This could reflect increased glucose tolerance in the 11βHSD-1$^{-/-}$ mice. Plasma insulin was highly variable but similar in all feeding states in the 2 genotypes.

Liver transcript profile of Fed 11βHSD-1$^{-/-}$ indicates normal lipid synthesis and increased lipid oxidation– To investigate the origins of the alterations in plasma lipids, expression of mRNAs encoding enzymes involved in the lipid synthetic (Fig. 3) and fatty acid oxidation pathways (Fig. 4) were examined by northern blot analysis. Fatty acid synthase (FAS) (Fig 3A) and glycerol-phosphate acyl transferase (GPAT) (Fig 3B), enzymes involved in triglyceride synthesis and esterification, respectively, were similarly expressed in 11ßHSD-1$^{-/-}$ and wild-type mice under *ad lib* fed conditions. Indeed levels of the crucial lipogenic transcription factor SREBP-1c that drives expression of FAS, GPAT and other enzymes in the lipid synthesis pathway (25, 26) were comparable between genotypes (Fig 3C). This implies that reduced triglyceride synthesis and esterification is unlikely to play a role in the lowered plasma triglyceride profile of 11βHSD-1$^{-/-}$ mice. Furthermore, mRNA encoding the rate-limiting enzyme in cholesterol synthesis, hydroxy-methyl-glutaryl-CoA-reductase (HMG-CoAR) was also expressed at similar levels in both genotypes in the fed state (Fig 3D).

In contrast, when enzymes of fatty acid oxidation were examined we found that mRNAs encoding carnitinepalmitoyl-transferase-I (CPT-I), a key rate-limiting enzyme in the mitochondrial β-oxidation pathway (27), acyl-CoA oxidase (ACO), a microsomal enzyme involved in fatty acid oxidation (28), and uncoupling protein-2 (UCP-2), a protein also implicated in the oxidation of fatty acids (29) and known to be expressed in hepatocytes (30), were all elevated in livers of fed 11βHSD-1$^{-/-}$ mice (Figs 4A, 4B, 4C). Moreover, PPARα mRNA, the key hepatic transcription factor regulating these genes of fatty acid oxidation was elevated in fed 11βHSD-1$^{-/-}$ mice (Fig 4D). Elevated expression of CPT-I, ACO and UCP-2 is consistent with these genes being downstream targets of PPARα (31), (32), (30, 33).

11βHSD-1$^{-/-}$ mice have an atheroprotective lipoprotein and fibrinogen profile – We also investigated the expression of glucocorticoid sensitive lipoproteins to further dissect the origin of the reduced triglyceride and increased HDL levels. Nephelometry was performed with specific anti-

apolipoprotein antibodies on a representative sample of serum from both genotypes in the fed state. Consistent with a cardioprotective reduction in circulating triglycerides, serum levels of apoCIII, a triglyceride-rich component of VLDL that plays a key role in determining plasma triglyceride levels (34), was markedly reduced in 11βHSD-1$^{-/-}$ mice (wild type 0.87±0.14 versus null 0.48±0.1 g/L). Apolipoprotein AI mRNA, encoding the major component of the HDL particle (35), was significantly elevated in fed 11βHSD-1$^{-/-}$ mouse liver (Fig. 5A), with elevated circulating plasma apoAI levels. Interestingly, serum apoAII, another lipoprotein associated with the HDL particle was reduced (wild type 0.53±0.1 versus null 0.28±0.1 g/L). Serum levels of apoB and apoE were not different between genotypes.

To assess a hepatic transcript unrelated to lipoproteins or lipid metabolism, we investigated Aα-fibrinogen mRNA, which encodes a glucocorticoid-sensitive plasma factor (36) that is an independent cardiovascular risk factor (37). Aα-fibrinogen transcript levels were reduced by 25% in fed 11βHSD-1$^{-/-}$ mice (Fig. 5B).

11βHSD-1$^{-/-}$ mice show attenuated induction of glucocorticoid-sensitive transcripts with fasting – Fasting causes a 2 fold induction of PPARα in wild type mice (Fig. 4D), consistent with reports that this transcription factor mediates glucocorticoid-induced fatty acid oxidation during fast (Kersten *et al.*, 1999, Leone *et al.*, 1999). However, whilst 11βHSD-1$^{-/-}$ liver PPARα levels were higher than wild type levels during *ad lib* fed conditions, fasting induction of PPARα mRNA was abolished in 11βHSD-1$^{-/-}$ animals (Fig 4D). Despite the abolished induction of PPARα, the downstream target genes ACO and UCP-2 showed a fasting induction. This induction was smaller relative to the wild type ad lib to fasting induction. Such a modest induction could reflect the presence of relatively elevated ad lib fed PPARα levels in mice being activated by the increased levels of endogenous PPARα activators, fatty acids, during fasting. The glucocorticoid-inducible transcript apoAI also shows an attenuated rise on fasting, compatible with reduced effective glucocorticoid levels in hepatocytes (Fig 5B). In agreement with an attenuated fasting response, a blunted fast-mediated repression of the lipid esterification enzyme GPAT is observed in null mice compared to wild type mice (Fig. 3B). Furthermore, oil red O histology of fasting 11βHSD-1$^{-/-}$ liver shows a marked accumulation of lipid in comparison to fasted wild-type liver (Fig 6B versus 6D) indicating an attenuation of lipid metabolism or efflux. However, lipid accumulation resolves similarly in both genotypes upon re-feeding (data not shown). Also, fasting induction of CPT-I (Fig. 4A) appears normal and fasting plasma glucose is not significantly different between genotypes. This implies that the attenuation of glucocorticoid effects on fatty acid oxidation and gluconeogenesis is not dramatic enough to cause hypoglycaemia after a 24 hour fast in the 11βHSD-1$^{-/-}$ mice.

11βHSD-1 does not respond acutely to fasting/re-feeding in wild-type mice – To determine that the difference between wild type and 11βHSD-1$^{-/-}$ mice were not merely due to feeding-related alterations in 11βHSD-1 activity, transcript levels and activity of the wild type 11βHSD-1 was measured across the experimental groups. Neither 11βHSD-1 mRNA or activity levels were affected by a 24 hour acute fast or subsequent re-feeding (Fig. 7A, 7B). Thus, whilst the enzyme is critical for regulating the active intracellular glucocorticoid level, it does not appear to be acutely regulated by either the increased corticosterone (wild type, ad lib fed 25.2±7.2 versus wild type fasting 222 ±76 nmol/L, $p< 0.05$). Further, 11βHSD-1 mRNA and activity is not affected by the reduced insulin levels associated with fasting (wild type, ad lib 3131±81 versus wild type fasting 564±36 ng/ml) or with the subsequent influx of insulin upon re-feeding (4 hour re-fed value 6052±654 ng/ml).

11βHSD-1 mice have increased hepatic insulin sensitivity upon re-feeding after fast – We have investigated hepatic insulin sensitivity by assessing the relative changes in insulin-sensitive

transcript levels upon re-feeding after a 24 hour fast. Northern analysis shows that insulin repressible transcripts such as CPT-I and UCP-2 were more markedly suppressed in 11βHSD-1$^{-/-}$ mice (Fig 4A and 4C) upon re-feeding. Conversely, insulin-inducible transcripts, such as those in the lipogenic (SREBP-1, FAS, GPAT) and cholesterologenic (HMG-CoAR) pathways, were more markedly induced in 11βHSD-1$^{-/-}$ mice upon re-feeding (Fig 3A-D).

11βHSD-1 mice have improved glucose tolerance – Studies of dynamic glucose disposal indicate that 11βHSD-1$^{-/-}$ mice have improved glycaemic control (Fig. 8). Taking into account the reduced zero-time glucose levels in the 11βHSD-1$^{-/-}$ mice after fasting which likely reflects the attenuated stress reaction in fasting glucose production (16), area under the curve for glucose levels in 11βHSD-1$^{-/-}$ mice indicates overall improved glucose disposal after an intraperitoneal glucose load compared to wild type. This is in keeping with improved hepatic insulin sensitivity.

Discussion

Glucocorticoids have been implicated in the development of several metabolic defects found in the Metabolic Syndrome. The importance of pre-receptor metabolism of glucocorticoids is clear for the 11ß-HSD2-mineralocorticoid receptor system in the distal nephron (7, 8). Any biological role of 11ß-HSD1, which has been proposed to regenerate active corticoids in sites of high expression such as liver, has been obscure. Here we show that complete functional loss of 11βHSD-1 by gene knockout promotes a 'cardioprotective' plasma lipid and lipoprotein phenotype, at least in part due to changes in expression of key enzymes and transcription factors in the liver.

Distinct phenotypic responses can be defined in the 11βHSD-$1^{-/-}$ mice, depending on dietary status. *Ad lib* fed 11βHSD-$1^{-/-}$ mice exhibit a 'favourable' lipid profile resulting from an apparent increase in hepatic oxidative drive and reduced levels of several markers associated with increased cardiovascular risk. Fasted 11βHSD-$1^{-/-}$ mice show attenuated glucocorticoid-inducible responses consistent with those observed in their carbohydrate metabolism (16). Re-feeding after fasting indicates 11βHSD-$1^{-/-}$ mice have increased hepatic insulin sensitivity. An advantageous metabolic profile is also supported by demonstration of improved glycaemic control in 11βHSD-$1^{-/-}$ mice.

In the *ad lib* fed state, 11βHSD-$1^{-/-}$ mice exhibit several features of a 'cardioprotective' lipid and lipoprotein phenotype. Plasma triglyceride levels are reduced and potentially beneficial HDL cholesterol is elevated. Moreover, 11βHSD-$1^{-/-}$ animals have reduced serum apoCIII. ApoCIII increases plasma triglycerides by inhibiting hepatic lipolysis (38) and interfering with transfer of triglycerides to the liver (34, 39). Reduction of apoCIII would in itself, therefore, contribute to reduced triglycerides. Indeed, apoCIII is positively correlated with cardiovascular disease risk (40). Analogously, null mice show increased ApoAI transcript levels, consistent with raised plasma HDL cholesterol. ApoAI is the main component of HDL and is negatively associated with cardiovascular risk (35).

It is unlikely that increased synthesis of triglyceride or cholesterol contributes to this phenotype as the expression of key rate-limiting lipogenic and cholesterologenic enzymes was unaffected, consistent with the finding that the lipogenic transcription factor SREBP1c mRNA was also maintained at wild type levels. In contrast, key enzymes of hepatic fatty acid oxidation were elevated in the 11ßHSD-1 null mice, compatible with increased hepatic catabolism of triglyceride as a mechanism driving the plasma changes seen. This in part may stem from elevated PPARα and is consistent with reports that the genes of fatty acid oxidation CPT-I (31), ACO (32), as well as UCP-2 (30, 33) are targets for PPARα in liver.

Indeed, a number of changes observed in the 11βHSD-$1^{-/-}$ mice suggest elevated PPARα levels may play a functional role in the atheroprotective phenotype. Thus, PPARα activation by fibrate ligands lowers plasma triglyceride and represses apoCIII (23) and Aα-fibrinogen expression (41). The 25% reduction in Aα-fibrinogen transcript levels observed in the11βHSD-$1^{-/-}$ mice is similar to the effect of fibrate administration and is consistent with this transcript being PPARα repressible (41). Since changes in Aα-transcript levels closely follow changes in plasma levels (41) we infer that the reduced transcript levels observed here would be likely to contribute to the overall atheroprotective profile of the 11βHSD-$1^{-/-}$ mouse. High fibrinogen levels are independently correlated with increased cardiovascular risk (37). It could be said, therefore, that the fed 11βHSD-$1^{-/-}$ animals mimic in part the phenotype of a fibrate treated animal.

11ßHSD-1 null mice show apparently lower intracellular glucocorticoid levels and action in the face of elevated basal and post-stress (eg fasting) plasma corticosterone levels (16). This underlines the importance of regeneration of corticosterone from 11-dehydrocorticosterone in determining

intracellular glucocorticoid effects. The lack of induction of PPARα with fasting is compatible with this notion, but it cannot explain the elevated fed PPARα levels. PPARα is induced by glucocorticoids (18) and follows a diurnal cycle that parallels the corticosterone rhythm (19). This implies that control of PPARα expression by glucocorticoid occurs not only in extreme conditions such as the stress-response to fasting but also during the normal diurnal cycle where glucocorticoid and insulin levels show more modest changes. One potential explanation for elevated PPARα expression at the diurnal nadir (8am) in 11βHSD-1$^{-/-}$ mice is that they have subtly elevated plasma corticosterone levels at this time (this study: wild type 25.2±7.2nmol/l versus 11βHSD-1$^{-/-}$ 47.5±7.8 nmol/L, $p<0.05$, in good agreement with our previous reports (16, 24). This results from somewhat impaired negative feedback upon the HPA axis normally amplified by 11βHSD-1 (16, 21). Interestingly, 11β-HSD-1$^{-/-}$ mice show a reduced intracellular glucocorticoid response in brain in the face of an exaggerated stress-mediated increase in plasma corticosterone (42 J.Y. C.K. personal communication?). This would imply that liver gene expression is perhaps less sensitive to the exquisite regulation of gene expression mediated by 11βHSD-1 in the brain and is more sensitive to the prevailing plasma corticosterone levels. However, levels of the glucocorticoid-sensitive hepatic binding protein CBG and liver GR binding are similar (24) in *ad lib* fed 11βHSD-1$^{-/-}$ mice and wild type mice in the morning. The lack of down-regulation of GR (43) and CBG (44) in 11βHSD-1$^{-/-}$ liver, in the face of elevated plasma corticosterone levels indicate that effective glucocorticoid action within the liver is indeed attenuated, suggesting that factors other than merely plasma corticosterone concentrations are responsible for the increased hepatic PPARα expression. PPARα is regulated by myriad factors including other steroids (45), lipids (46), retinoids (47) and hormones (48), including insulin as shown in the present study. The precise determinants of elevated basal PPARα in this model of chronic subtle glucocorticoid depletion in the liver remain to be determined.

It is also clear that PPARα and GR have overlapping and sometimes opposing actions on target promoters. For example, fibrinogen levels are positively regulated by glucocorticoids (36, 49) and negatively regulated by PPARα (41). Similarly, apolipoprotein AI is induced by glucocorticoids (50) whereas in mice apoAI (23), and apoAII ( ) levels are repressed by PPARα. Our observation of elevated ApoAI transcript levels in fed 11βHSD-1$^{-/-}$ mice could imply that the apoAI promoter is more sensitive to glucocorticoid-mediated induction than to PPARα-mediated repression. For some promoters the GR effect seems to predominate, for others PPARα. Alternatively, since insulin is known to positively regulate the apoAI promoter (Murao et al., 1998), increased insulin sensitivity in 11βHSD-1$^{-/-}$ liver mice may also explain the discrepancy in gene expression observed. Further work will be necessary to determine the underlying mechanism for the apoAI expression pattern. However we would expect that since this component of the HDL reverse cholesterol transport system is negatively correlated with cardiovascular risk ( ) that elevated levels could contribute to the overall atheroprotective profile of 11βHSD-1$^{-/-}$ mice.

Among the physiological roles of glucocorticoids is the adaptation of animals to prolonged nutrient deprivation. During this response, elevated glucocorticoid levels drive increased hepatic glucose production and fatty acid oxidation whilst concomitantly facilitating adipose tissue lipolysis to provide the fatty acids and glycerol required by the liver. In the fasted state, 11ß-HSD-1$^{-/-}$ mice show attenuation of glucocorticoid-sensitive gene expression. PPARα and apoAI show attenuated induction whereas GPAT exhibits an attenuated fasting repression. These results are in agreement with previous findings on attenuation of glucocorticoid-inducible glucose metabolism in 11ß-HSD-1$^{-/-}$ mice (Kotelevtsev *et al.*, 1997). This implies that the null mice have a relative lack of intracellular glucocorticoid during fasting or stress. Despite this attenuated induction, the mice appear to be capable of maintaining their hepatic fatty acid oxidation system over a 24 hour fast. Thus, despite an abolished fasting induction of PPARα in11ß-HSD-1$^{-/-}$ mice, a major rate limiting enzyme in mitochondrial oxidation (CPT-I) appears to be normally induced, and fasting plasma

glucose levels are not significantly lower than wild type animals. This is in contrast to findings in fasted PPARα null mice which exhibit profound hypoglycaemia upon prolonged fasting (Kersten et al., 1999, Leone et al., 1999). There is relatively pronounced lipid accumulation in 11βHSD-1$^{-/-}$ liver on fasting, reminiscent of the fatty liver observed in fasted PPARα null mice (Kersten et al., 1999, Leone et al., 1999). However, lipid accumulation seems to resolve in 11ß-HSD-1$^{-/-}$ mice upon re-feeding. Whether lipid accumulation is due to blunted PPARα-driven increases in fatty acid oxidation, as in fasted PPARα knockout mice, remains to be determined. Indeed, whilst PPARα may regulate CPT-I levels in the *ad lib* state (Yu et al., 1998), fast-mediated induction of CPT-I is unaffected in PPARα knockout mice (Le May *et al.*, 2000) implying that this process is independent of the transcription factor. An alternative explanation could come from our observation of attenuated glucocorticoid-mediated fasting repression of the lipid esterification enzyme GPAT. Elevated levels of such a rate limiting enzyme in the lipid synthesis pathway could contribute to the lipid accumulation observed. Indeed, raised GPAT levels may also partly account for the lower fold reduction in plasma triglyceride on fasting in null mice compared to wild type. Since GPAT is insulin-inducible, this finding is also consistent with the growing evidence that 11βHSD-1$^{-/-}$ liver is more sensitive than wild type to even the extremely low insulin levels found during fasting. The PPARα-sensitive ACO and UCP-2 transcripts show attenuated induction with fasting and may reflect the relatively greater sensitivity of these promoters, compared to that of CPT-I, to PPARα regulation on fasting. Partial induction of downstream target genes by PPARα in the face of a blunt fasting increase in PPARα levels could mean that activation of the already elevated 11βHSD-1$^{-/-}$ levels of PPARα within a 24-hour fasting period is sufficient to promote an adaptive metabolic response in 11βHSD-1$^{-/-}$ mice. This is a possibility since endogenous fatty acids activate PPARα (Forman et al., 1997) and there is an increased provision of fatty acid to the liver during a fast. Alternatively, other processes may elevate expression of the oxidative enzymes during fasting (Le May et al., 2000).

Re-feeding after a fast is characterised by a pronounced insulin-mediated overshoot in liver gene expression of enzymes in the lipogenic pathways and repression of oxidative processes. We have used this as a measure of hepatic insulin sensitivity. 11βHSD-1$^{-/-}$ mice clearly have increased hepatic insulin sensitivity since on refeeding there is an exaggerated suppression (CPT-I, UCP-2) or induction (SREBP-1c, FAS, GPAT, HMGCoA-R) of transcript levels for oxidative and lipogenic enzymes, respectively. Pronounced induction of the lipogenic pathway (SREBP-1c, FAS and GPAT) combined with an exaggerated repression of oxidative lipid metabolism (CPT-I, UCP-2) upon re-feeding after fast may also account for the rapid return of triglycerides to *ad lib* fed values by 4h and the overshoot of plasma triglycerides seen at the 24 hour re-feeding period in 11βHSD-1$^{-/-}$ mice. The contention of increased insulin sensitivity is supported by intraperitoneal glucose tolerance tests that show 11βHSD-1$^{-/-}$ mice have improved glycaemic control. However, muscle is the major post-prandial site of glucose disposal, and it is unclear whether improved insulin sensitivity in the liver of the 11βHSD-1$^{-/-}$ mice can account entirely for the improved glucose tolerance. Direct studies on 11βHSD-1$^{-/-}$ mouse muscle are required to address this issue. Clearly, since insulin resistance is one of the major underlying mechanisms ascribed to the pathogenic development of the metabolic syndrome, demonstration of increased hepatic insulin sensitivity and improved glucose tolerance can be interpreted as beneficial.

Mice with a targeted disruption in the gene encoding the 11βHSD-1 enzyme represents a model animal that lacks a crucial intracellular glucocorticoid re-amplifying mechanism. 11βHSD-1$^{-/-}$ mice resist hyperglycaemia upon stress and obesity ( ) and have a favourable metabolic and lipidaemic profile due to altered expression and activity of liver proteins. However, 11βHSD-1 is also expressed in other tissues such as fat and brain, important sites regulating lipid and nutrient homeostasis. 11βHSD-1 may also, therefore, modulate glucocorticoid action on central energy

balance as well as peripheral fat storage, insulin action and glucose tolerance. These effects cannot be ruled out as having a bearing on the lipid profile, in combination with the hepatic effects of 11βHSD-1 knockout on lipid metabolism described here. Nevertheless, the improved fed and re-fed metabolic profiles in the 11ßHSD-1 null mice suggest inhibitors of this enzyme may have favourable effects on several cardiovascular risk factors. This is particularly pertinent as the expression of the enzyme in liver was unaffected by the dietary manipulations *in vivo*, suggesting that the putative drug target is maintained. Further, a combination of an 11βHSD-1 inhibitor and a fibrate could represent an extremely powerful therapeutic strategy for treating dyslipidaemias, glucose intolerance and hyperfibrinogenaemia.

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Figure legends

Figure 1. ***A***. Triglyceride levels in wild type (solid bars) versus 11βHSD-1$^{-/-}$ (open bars) animals subjected to dietary manipulation: **AL**; ad lib fed, **F**; 24h fasted, **4RF**; 24h fast with 4h re-feed and **24RF**; 24h fast with 24h re-feed. ***B***. Representative true triglyceride FPLC profile from *ad lib* fed wild type ( ) and 11βHSD-1$^{-/-}$ mice.

Figure 2. ***A.*** Total cholesterol levels in wild type (solid bars) versus 11βHSD-1$^{-/-}$ (open bars) animals subjected to dietary manipulation: **AL**; ad lib fed, **F**; 24h fasted, **4RF**; 24h fast with 4h re-feed and **24RF**; 24h fast with 24h re-feed. ***B.*** HDL cholesterol levels in wild type (solid bars) versus 11βHSD-1$^{-/-}$ (open bars) animals subjected to dietary manipulation: **AL**; ad lib fed, **F**; 24h fasted, **4RF**; 24h fast with 4h re-feed and **24RF**; 24h fast with 24h re-feed.

Figure 3. Transcript levels of proteins of the lipogenic (***A-C***) and cholesterol biosynthesis pathways (***D***) in livers of wild type (solid bars) versus 11βHSD-1$^{-/-}$ (open bars) animals subjected to dietary manipulation: **AL**; ad lib fed, **F**; 24h fasted, **4RF**; 24h fast with 4h re-feed and **24RF**; 24h fast with 24h re-feed. Transcript levels were analysed by northern blot as described in Materials and Methods. ***A***. Fatty acid synthase tarnscript levels. ***B.*** Glycerolphosphate acyl transferase transcript levels. ***C.*** Sterol regulatory element binding protein-1c transcript levels. ***D.*** Hydroxy-methyl-glutaryl CoA synthase transcript levels. Transcript levels were corrected for RNA loading by using a cDNA probe for the U1 small ribonucleoprotein.

Figure 4. Transcript levels of proteins in the fatty acid oxidation pathway in livers of wild type (solid bars) versus 11βHSD-1$^{-/-}$ (open bars) animals subjected to dietary manipulation: **AL**; ad lib fed, **F**; 24h fasted, **4RF**; 24h fast with 4h re-feed and **24RF**; 24h fast with 24h re-feed. Transcript levels were analysed by northern blot as described in Materials and Methods. ***A***. Carnitinepalmitoyltransferase-I (CPT-I) transcript levels. ***B.*** Acyl coA oxidase transcript levels. ***C.*** Uncoupling protein-2 transcript levels. ***D.*** Peroxisome proliferator-activated receptor-α transcript levels. Transcript levels were corrected for RNA loading by using a cDNA probe for the U1 small ribonucleoprotein.

Figure 5. Transcript levels for glucocorticoid-sensitive markers of cardiovascular risk in livers of wild type (solid bars) versus 11βHSD-1$^{-/-}$ (open bars) animals subjected to: **AL**; ad lib feeding or **F**; a 24h fast. Transcript levels were analysed by northern blot as described in Materials and Methods. ***A***. The HDL component apolipoproteinAI transcript levels. ***B***. Independent cardiovascular risk factor, Aα-fibrinogen, transcript levels. Transcript levels were corrected for RNA loading by using a cDNA probe for the U1 small ribonucleoprotein.

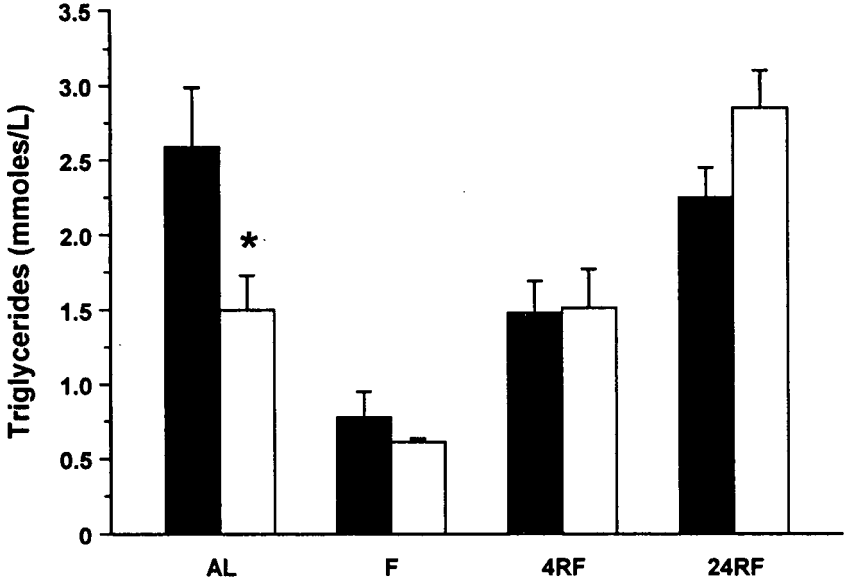
Figure 6. Oil red O histology of liver sections of wild type (solid bars) versus 11βHSD-1$^{-/-}$ (open bars) animals subjected to dietary manipulation: **AL**; ad lib fed, **F**; 24h fast. Liver sections (20μm) were stained with Oil red O as described in Experimental Procedures. ***A.*** wild type *ad lib* fed liver. ***B***. wild type liver after 24h fast ***C***. 11βHSD-1$^{-/-}$ *ad lib* fed liver.***D***. 11βHSD-1$^{-/-}$ liver after 24h fast.

Figure 7. Transcript and activity levels of 11βHSD-1 in livers of wild type animals subjected to dietary manipulation: **AL**; ad lib fed, **F**; 24h fasted, **4RF**; 24h fast with 4h re-feed and **24RF**; 24h fast with 24h re-feed. Transcript levels were analysed by northern blot as described in Materials and Methods. Activity levels were determined in homogenates of liver as described in Materials and Methods. ***A.*** 11βHSD-1 transcript levels. ***B.*** 11βHSD-1 Activity.

Figure 8. Intraperitoneal glucose tolerance test in overnight fasted wild type (solid circles) versus 11βHSD-1$^{-/-}$ mice (open squares).

Figure 1

A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B

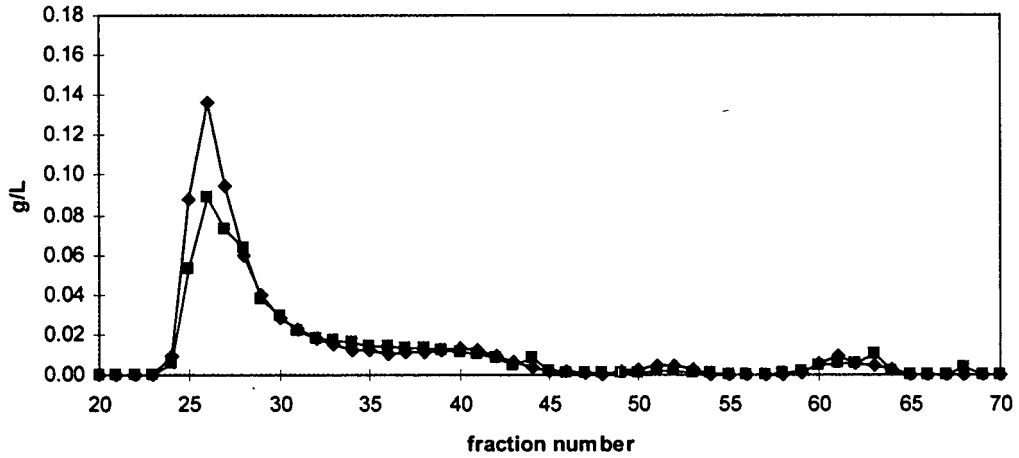
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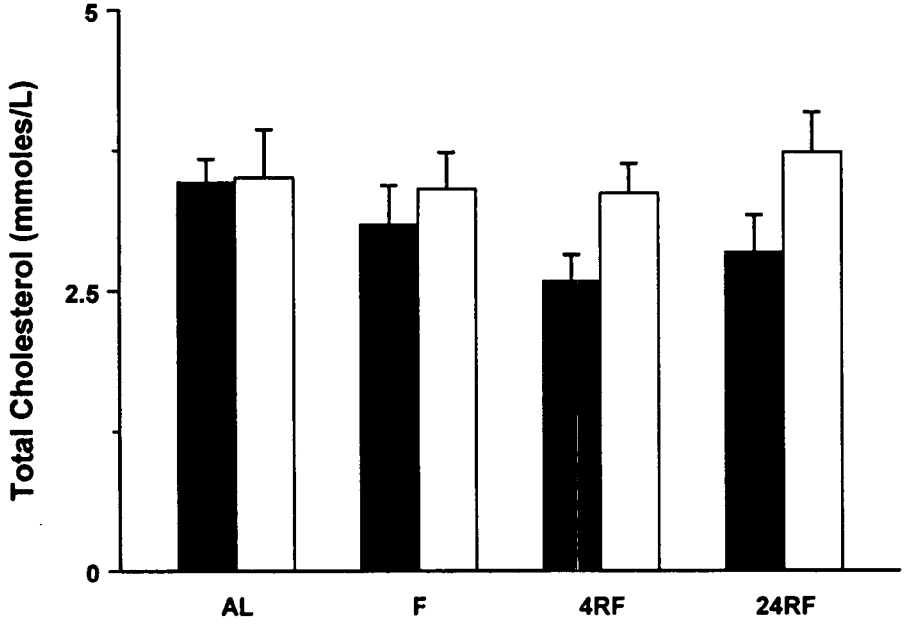
Figure 2

A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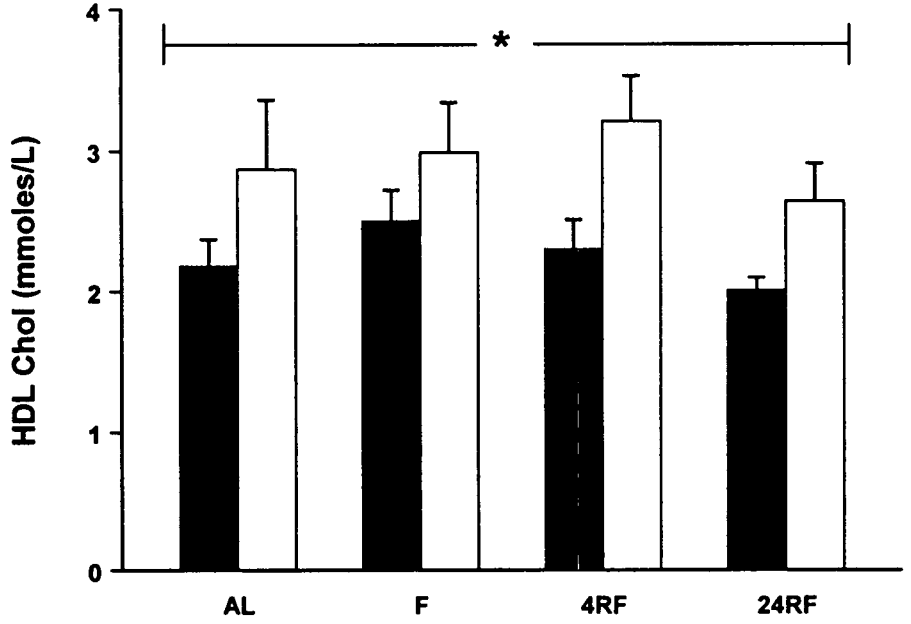
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HDL Chol (mmoles/L)
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Figure 3

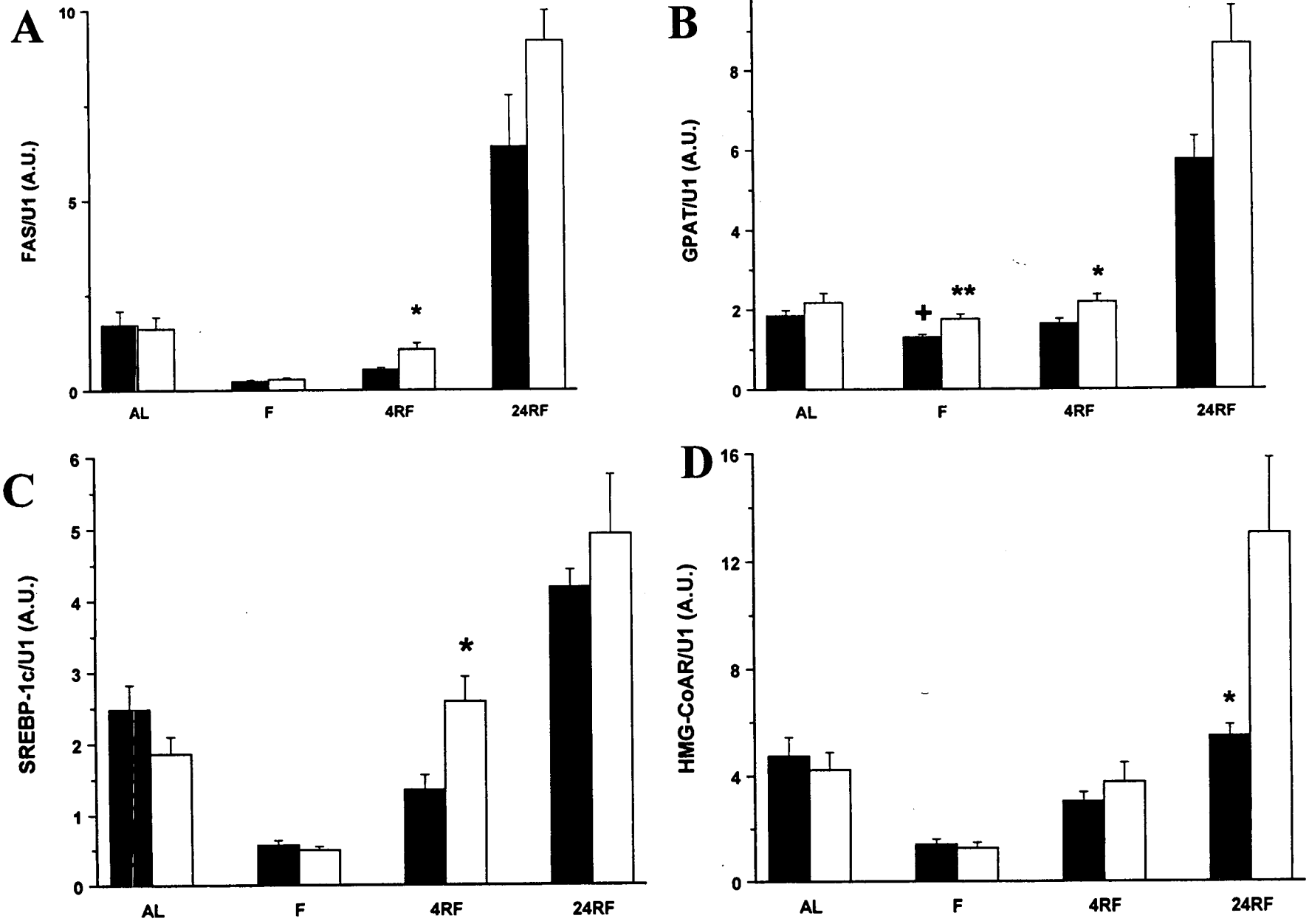
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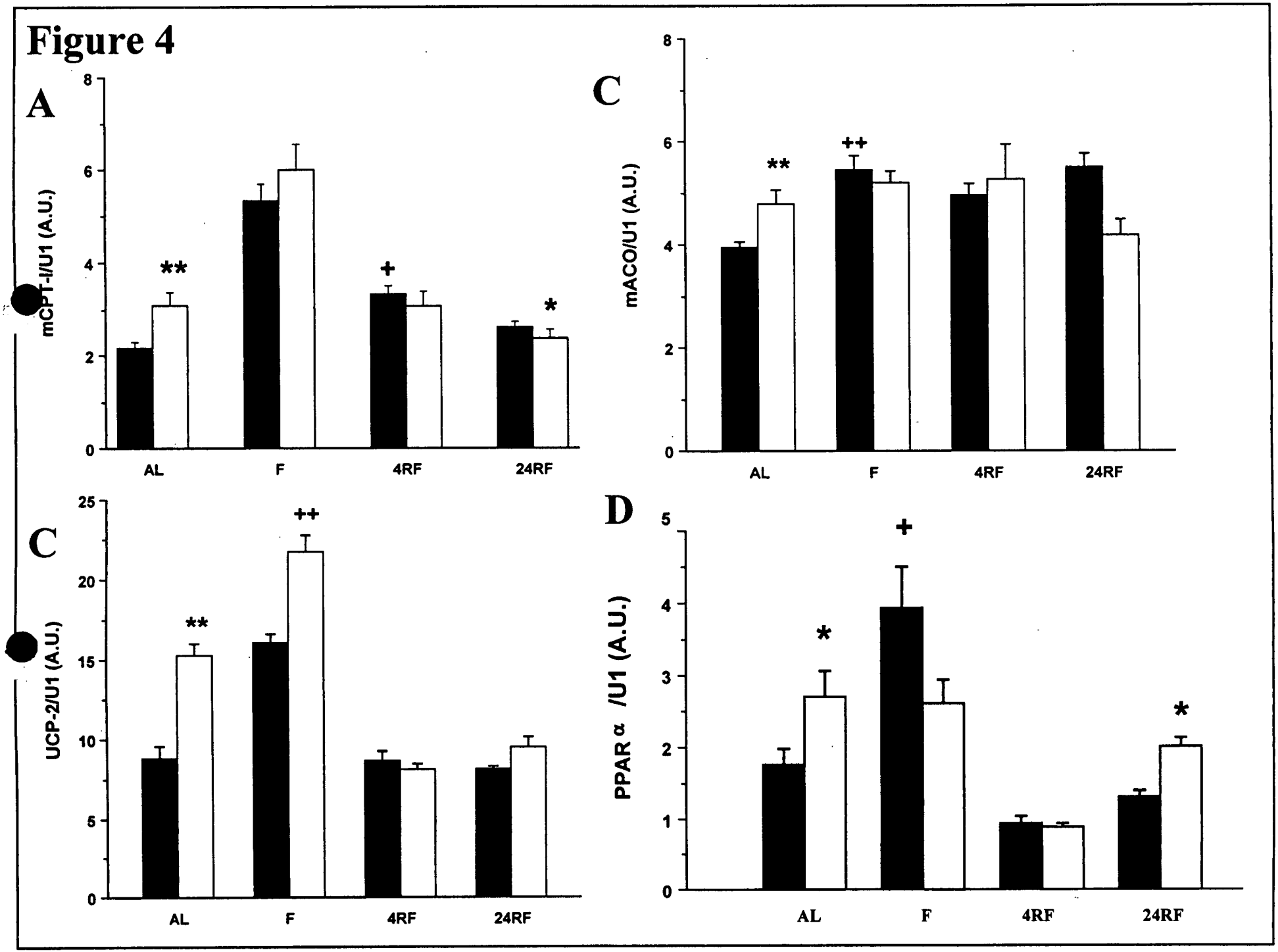
Figure 4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F
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Figure 5

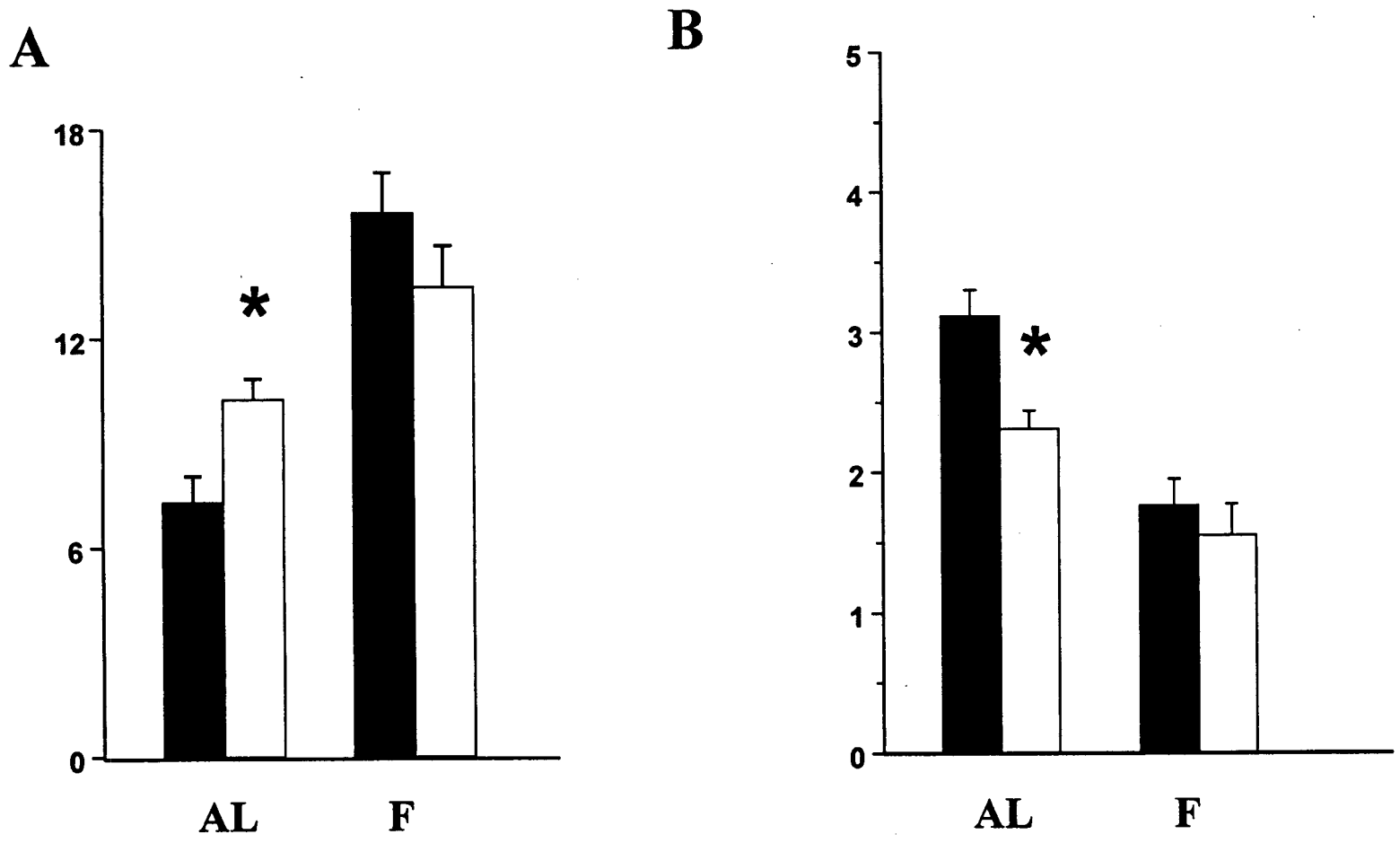


Figure 6

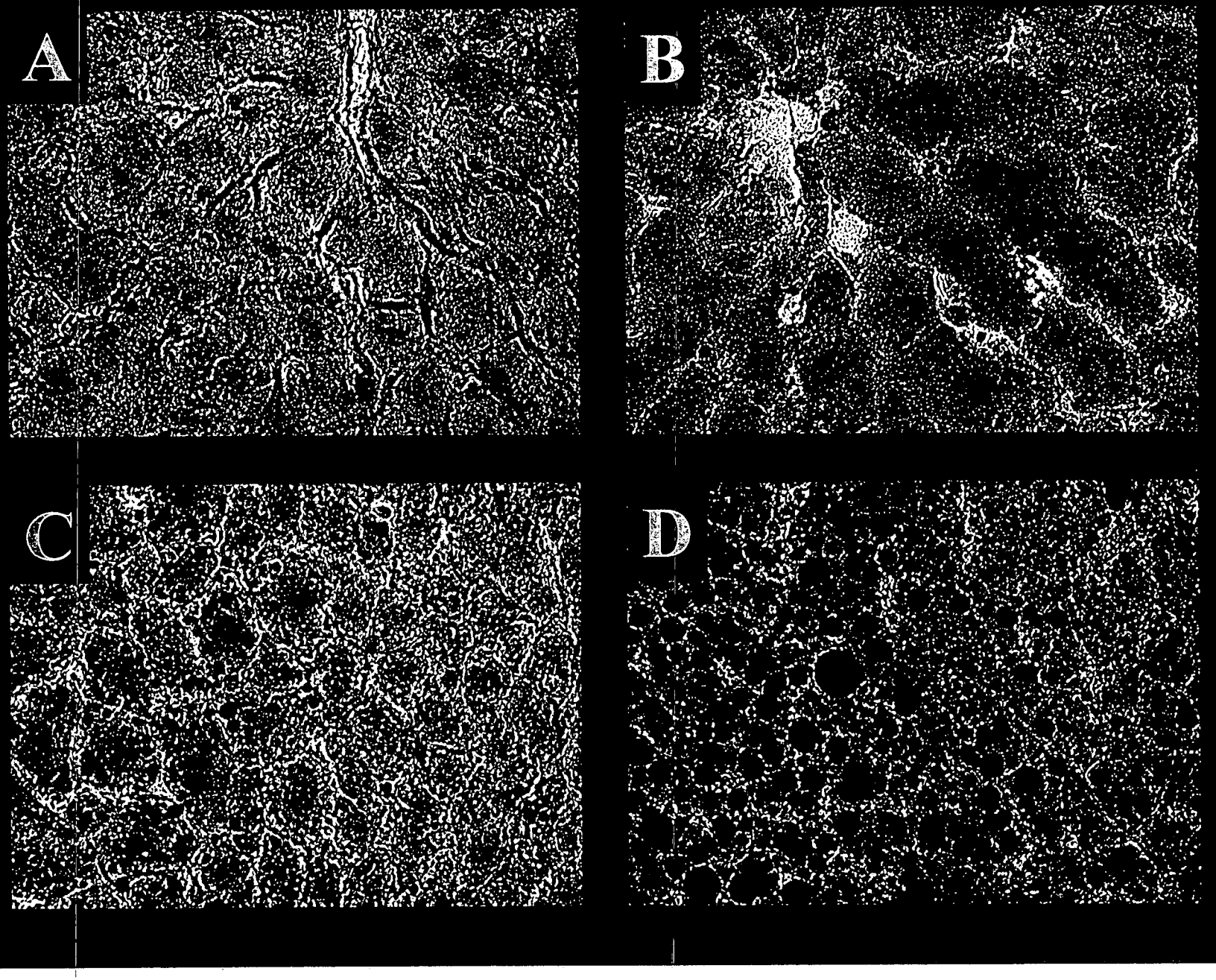


Figure 7

A

mRNA blot

B

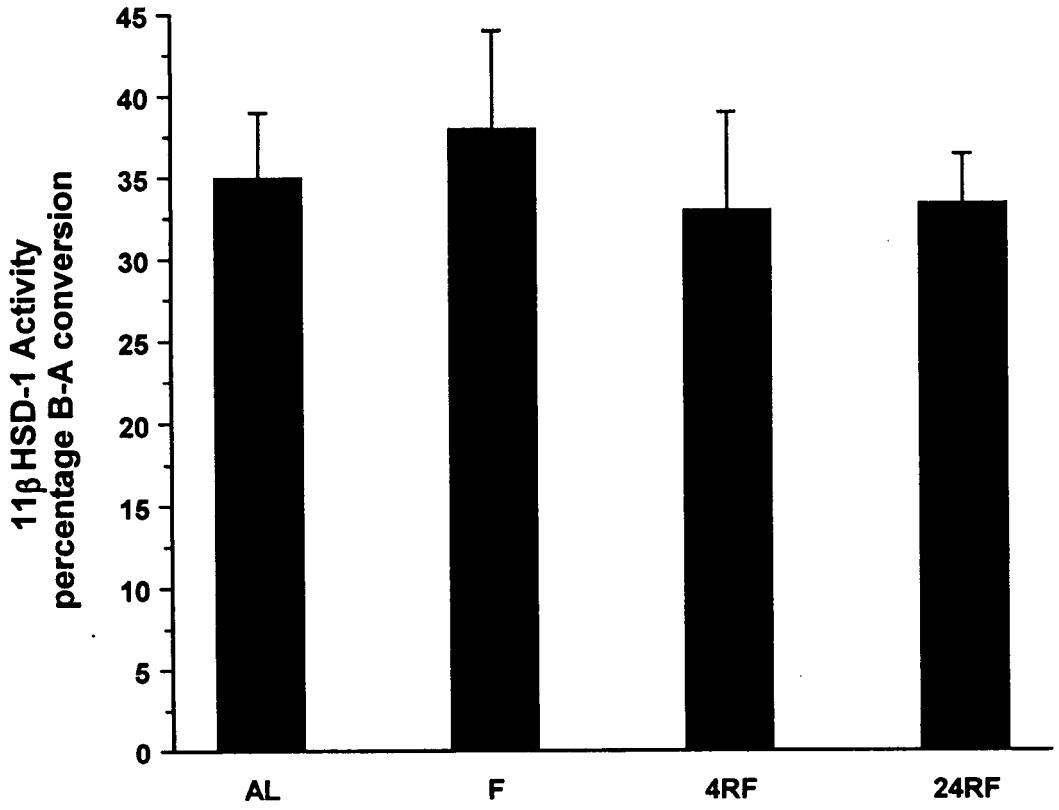
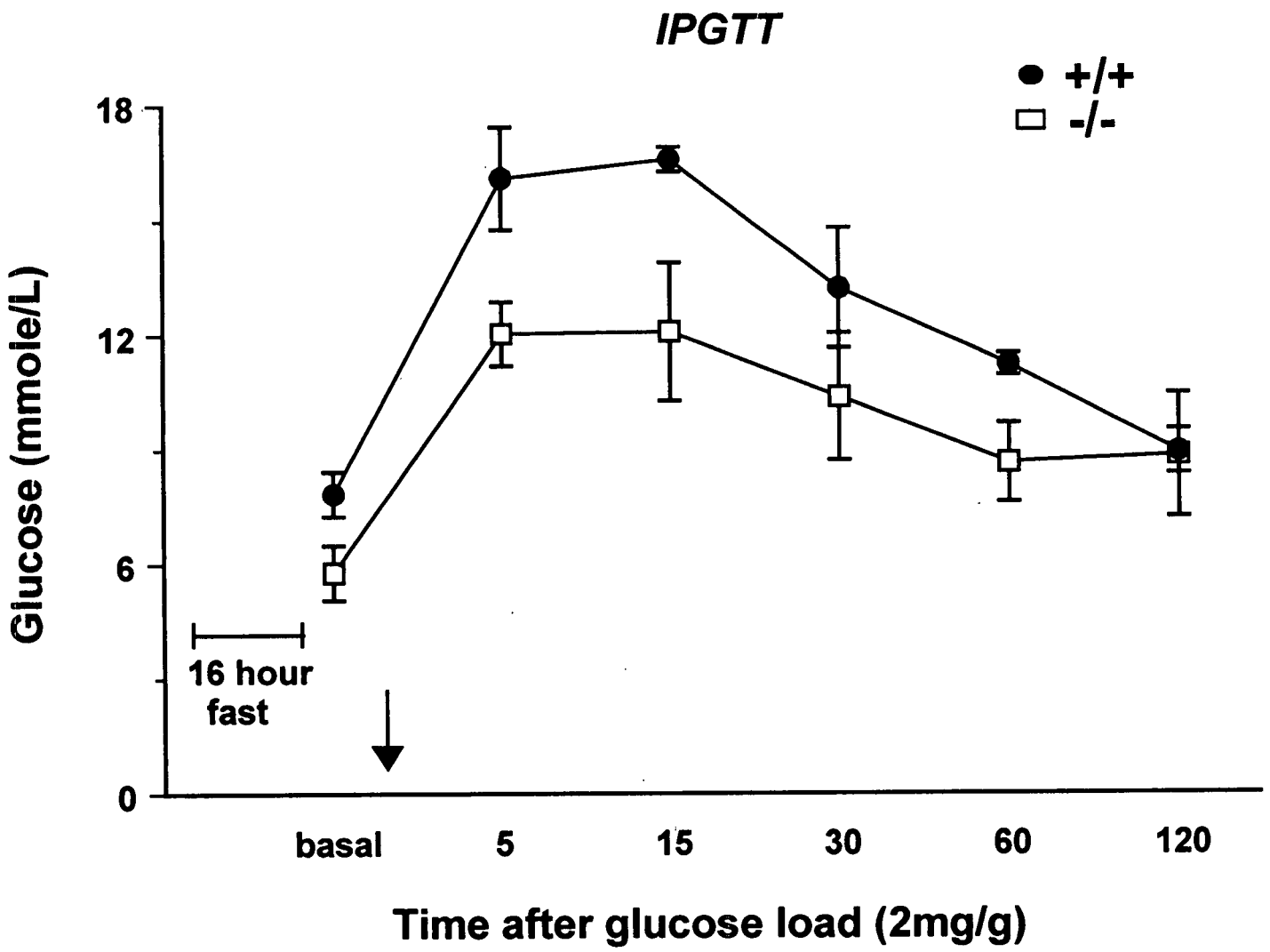


Figure 8


IPGTT
+/+
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18
12
6
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Glucose (mmole/L)
16 hour
fast
basal
5
15
30
60
120
Time after glucose load (2mg/g)

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11β-Hydroxysteroid Dehydrogenase in Cultured Hippocampal Cells Reactivates Inert 11-Dehydrocorticosterone, Potentiating Neurotoxicity

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11β-Hydroxysteroid dehydrogenase (11β-HSD) catalyzes the conversion of the glucocorticoid corticosterone (cortisol in humans) to inert 11-dehydrocorticosterone (cortisone). 11β-HSD activity is present in the hippocampus, where it is induced by glucocorticoids and stress *in vivo*, prompting suggestions that the enzyme may attenuate the deleterious effects of chronic glucocorticoid excess on neuronal function and survival. Two isoforms exist: 11β-HSD1, a bidirectional NADPH-dependent enzyme, and 11β-HSD2, an NAD^+-dependent exclusive 11β-dehydrogenase (corticosterone-inactivating enzyme). In this study, 11β-HSD1 activity and mRNA synthesis were demonstrated in primary fetal hippocampal cell cultures. Unexpectedly, the reaction direction in intact hippocampal cells was 11β-reduction (reactivation of inert 11-dehydrocorticosterone), although homogenization revealed that the enzyme was capable of 11β-dehydrogenation when removed from its normal cellular context. Dexamethasone (10^{-7} M) increased 11β-HSD activity in homogenates of hippocampal cultures (102% increase). In intact hippocampal cells, dexamethasone induced 11βreductase, not dehydrogenase. To determine the functional relevance of hippocampal 11β-reductase, glucocorticoid potentiation of kainic acid neurotoxicity was examined. Pretreatment of hippocampal cells with corticosterone reduced survival on kainate exposure. Hippocampal cell 11β-HSD activity was potently inhibited by carbenoxolone. Carbenoxolone had no effect on cell survival after kainate alone and did not alter the effect of corticosterone. 11-Dehydrocorticosterone also potentiated kainate neurotoxicity; this effect was lost, however, if 11β-HSD was inhibited with carbenoxolone. Thus, hippocampal 11β-HSD seems to be a functional 11β-reductase in intact cells. Measures to attenuate hippocampal 11β-reductase may reduce neuronal vulnerability to glucocorticoid toxicity.

Key words: NADPH; dexamethasone; glucocorticoids; carbenoxolone; corticosterone; kainic acid

11β-Hydroxysteroid dehydrogenase (11β-HSD) catalyzes the conversion of physiological glucocorticoids (corticosterone, cortisol) to inert 11-keto derivatives (11-dehydrocorticosterone, cortisone) (Monder and White, 1993). *In vivo*, 11β-HSD ensures selective access of aldosterone over corticosterone to mineralocorticoid receptors (MRs) in the distal nephron (Edwards et al., 1988; Funder et al., 1988); MRs are otherwise nonselective and bind corticosterone with similar affinity to aldosterone *in vitro* (Arriza et al., 1987, 1988). When 11β-HSD is congenitally absent or inhibited by licorice (or its derivative carbenoxolone), glucocorticoids illicitly occupy renal MRs, causing sodium retention and hypertension (Stewart et al., 1987, 1988, 1990).

Glucocorticoids, which are released from the adrenal cortex in response to circadian or stress-induced activation of the hypothalamic–pituitary–adrenal axis, subserve many roles in homeostasis and the response to stress. The brain is a key target for glucocorticoid action, which is mediated via both MRs and lower affinity glucocorticoid receptors (McEwen et al., 1986a; de Kloet, 1991; Seckl and Olsson, 1995). The hippocampus expresses a higher density of MRs than does the kidney, but these sites are occupied by corticosterone *in vivo* (de Kloet et al., 1975; Reul and de Kloet, 1985; McEwen et al., 1986a; de Kloet, 1991), suggesting that 11β-HSD is absent. However, several recent studies have demonstrated 11β-HSD activity, immunoreactivity, and mRNA expression in hippocampal cells (neurons) (Moisan et al., 1990; Lakshmi et al., 1991; Sakai et al., 1992), raising the possibility of an aldosterone-selective subset of hippocampal MRs (Moisan et al., 1990). Indeed, some data suggest that a proportion of hippocampal aldosterone binding is not readily displaced by corticosterone (McEwen et al., 1986b) and that not all functions of aldosterone and corticosterone in the hippocampus are identical (de Kloet et al., 1983). Administration of 11β-HSD inhibitors alters functional activity in the hippocampus *in vivo* (Seckl et al., 1991), although the mechanisms underpinning this effect are obscure. Hippocampal 11β-HSD is induced by chronic glucocorticoid excess or stress (Low et al., 1994b). Because chronic glucocorticoid excess exerts well documented deleterious actions on hippocampal cell function and survival (Landfield et al., 1978; Sapolsky et al., 1985, 1986; Sapolsky, 1992), it has been postulated that such induction of 11β-HSD is protective (Monder, 1991; Low et al., 1994b; Seckl and Olsson, 1995). Nevertheless, the presence of 11β-HSD activity in the hippocampus contradicts a majority of data, which indicate nonselective MRs at this site.

Recently, it has become apparent that there are at least two distinct 11β-HSD isoforms (Seckl, 1993). Target organs for aldosterone and the placenta express a high-affinity, NAD^+-dependent enzyme (11β-HSD2), which is an exclusive 11β-dehydrogenase (corticosterone inactivating enzyme) (Brown et al., 1993; Albiston et al., 1994). In contrast, the liver-derived isoform (11β-HSD1) is

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a lower-affinity, $NADP^+$/NADPH-dependent enzyme (Lakshmi and Monder, 1988; Agarwal et al., 1989). Expression of 11β-HSD1 cDNA in a range of cell lines encodes either a bidirectional enzyme (Agarwal et al., 1989) or a predominant 11β-reductase (Duperrex et al., 1993; Low et al., 1994a). 11β-Reductase activity, best observed in intact cells, activates 11-dehydrocorticosterone to alter target gene transcription and differentiated cell function (Duperrex et al., 1993; Low et al., 1994b). In homogenates of hippocampus, both dehydrogenation and reduction occur (Lakshmi et al., 1991), but the reaction direction in intact cells is unknown. We therefore have examined 11β-HSD activity and its function in primary cultures of fetal hippocampal cells.

MATERIALS AND METHODS

Cell culture media were obtained from Gibco (Paisley, UK); corticosterone (B), 11-dehydrocorticosterone (A), poly-D-lysine, insulin, apo-transferrin, putrescine, sodium selenite, and progesterone were obtained from Sigma (Poole, UK). Tissue culture plastics were from Costar UK Ltd (High Wycombe, Bucks, UK). [^{3}H]-1,2,6,7-Corticosterone ([^{3}H]B; ~ 72 Ci/mmol) was obtained from Amersham International (Aylesbury, Bucks, UK). [^{3}H]11-Dehydrocorticosterone ([^{3}H]A) was prepared by incubating [^{3}H]B with human placental extract, a concentrated source of 11β-dehydrogenase (11β-HSD2), as described previously (Low et al., 1994a). Purity was typically >99%, monitored on HPLC.

Primary hippocampal neuronal culture. The cell cultures were prepared according to a method derived from Mitchell et al. (1990). The medium (pH 7.3) contained DMEM with Glutamax-I (0.086%), 10% fetal bovine serum (FBS) or donor horse serum (DHS), 15 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin and glucose increased to 0.2%. Day 18 fetuses were removed by laparatomy, and the hippocampi were dissected into HBSS containing 15 mM HEPES, pH 7.4. The cells were incubated in trypsin-EDTA for 20 min, washed, and mechanically dissociated by trituration. Cells were seeded at a density of 0.8–1.0 × 10^6 cells/ml of medium and plated on 35 mm Petri dishes previously coated with 0.025 mg/ml poly-D-lysine. The cells were cultured in a water-saturated atmosphere at 37°C, 10% CO_2, for 3 d in the presence of serum, and then changed to defined (serum-free) medium (DMEM-F12 containing Glutamax-I, 10 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 20 nM sodium selenite, 20 nM progesterone, 100 IU/ml penicillin, and 100 μg/ml streptomycin). The cells were maintained in this medium for 5 d with one-third of the medium changed every 3 d until experimentation. Under these conditions, the cultures contained 60–80% neurons with glia forming the remainder, as determined by immunostaining of specimen cultures with antisera to glial fibrillary axial protein and neuron-specific enolase.

11β-HSD activity. 11β-Reductase and 11β-dehydrogenase activity were determined in intact primary hippocampal cells by the addition of 25 nM [^{3}H]A or [^{3}H]B to the medium, as described previously (Low et al., 1994a). Aliquots of the culture medium were removed at intervals over 24 hr and put into ethyl acetate; the steroids were extracted, dried under N_2, and suspended in 100 ml of ethanol containing 2.5 mg/ml cold A and B. Steroids were separated by thin-layer chromatography (TLC) in chloroform/95% ethanol (92:8), and bands were visualized under ultraviolet light and scraped into scintillation vials containing 3 ml of liquid scintillant (Cocktail T, BDH, Lutterworth, UK). The radioactivity in each fraction was determined, and enzyme activity was expressed as the percentage conversion to reaction product (Low et al., 1994a). Blanks, ^{3}H-labeled steroids incubated in medium on dishes without cells and extracted as above, were subtracted.

11β-Reductase and 11β-dehydrogenase activity also were determined in homogenates of cultured hippocampal cells, broadly as described previously (Moisan et al., 1990; Low et al., 1994a). Cells were washed with PBS and homogenized in Buffer C [20 mM tricine, 1.07 mM $(MgCO_3)_4 \cdot Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, and 0.2 mg/ml coenzyme A] containing 0.1% Triton X-100. Protein was measured by Bradford's method (Bio-Rad protein assay kit, Bio-Rad, Hemel Hempstead, UK). Preliminary studies established conditions such that the amount of protein added was within the linear portion of the relationship between protein concentration and percentage substrate conversion. Thus, an aliquot of hippocampal cell homogenate (typically 64 μg protein/ml) was incubated at 37°C for 60 min with 10 nM [^{3}H]B (11β-dehydrogenase) or [^{3}H]A (11β-reductase), 400 μM $NADP^+$ or NADPH, and PBS to a total volume of 50 μl. Steroids were extracted and separated by TLC. and enzyme activity was calculated. Blanks were subtracted.

11β-HSD mRNA analysis. Expression of 11β-HSD1 and 11β-HSD2 mRNA was determined by PCR. In brief, total RNA was extracted from cell homogenates with guanidinium thiocyanate (Chomczynski and Sacchi, 1987). One microgram of RNA was subjected to reverse transcription in a total volume of 20 μl using the Promega Reverse transcription kit (Promega, Southampton, UK). A 2 μl aliquot of the reverse transcription reaction was used in the PCR reaction after denaturation at 96°C for 10 min. The PCR mix contained 1.5 mM $MgCl_2$, 40 μM dNTPs, 20 pmol of each primer, and 2.5 U of *Taq* DNA polymerase in a final volume of 50 μl. The primer pairs for 11β-HSD1 were 869P (5'-AAAGCTTGTCACA/TGGGGCCAGCAAA-3'), corresponding to nucleotides 178–207 of rat 11β-HSD1 cDNA, and 868P (5'-AGGATCCAG/AAGCAAACTTGCT-TGCA-3'), complementary to nucleotides 648–628 of rat 11β-HSD1 cDNA. The primer pairs for 11β-HSD2 were P2520 (5'-CAATGCTG-GCCTCAACATGGT-3'), corresponding to nucleotides 624–644 of the rat 11β-HSD2 cDNA, and P2521 (5'-GGTCCTGGGTTGTGTCAT-GAA-3'), complementary to nucleotides 1297–1277 of the rat 11β-HSD2 cDNA sequence (Zhou et al., 1995). Both sets of primers span intron sequences to distinguish DNA products (>4 kb for 11β-HSD1, ~0.93 kb for 11β-HSD2) from mRNA products (0.43 kb for 11β-HSD1, 0.63 kb for 11β-HSD2). The PCR conditions were 30 cycles of 96°C for 30 sec, 50°C for 45 sec, and 72°C for 90 sec, followed by extension at 72°C for 10 min.

Modulation of 11β-HSD activity in primary hippocampal cells. To determine whether 11β-HSD activity in intact hippocampal cells was inhibited by licorice derivatives, cultures were pretreated with carbenoxolone (10^{-6} M) for 24 hr, and enzyme activity in 11β-reductase and 11β-dehydrogenase directions was estimated, as above. Glucocorticoid effects on enzyme activity were determined by preincubation of cultured hippocampal cells with dexamethasone (10^{-7} M) for 72 hr before enzyme measurement in intact cells after 8 hr incubation with ^{3}H-labeled steroids (a period chosen to reflect submaximal 11β-reductase and detectable 11β-dehydrogenase activities).

Effects of 11β-HSD on hippocampal cell vulnerability to kainate toxicity. To determine the functional relevance of 11β-HSD activity in primary cultures of hippocampal cells, the effects of corticosterone and 11-dehydrocorticosterone in the presence or absence of carbenoxolone on cell survival in response to kainic acid stimulation were determined, as described previously (Sapolsky, 1986; Packan and Sapolsky, 1990). In brief, cells were cultured as described above, but in the presence of 2% FBS to improve cell survival in the presence of kainic acid. This did not alter the predominant 11β-reduction in intact hippocampal cells or affect enzyme activity levels: corticosteroid levels are $<10^{-10}$ M in FBS (S.C. Low and J. R. Seckl, unpublished observations.) Plates of cells were pre-exposed to 11-dehydrocorticosterone (10^{-5} M) or corticosterone (10^{-5} M) in the presence or absence of carbenoxolone (10^{-6} M) for 24 hr. Controls included cultures exposed to carbenoxolone alone and cultures to which nothing was added. All cells then received kainic acid (10^{-5} M). After 48 hr, cells were washed in PBS, scraped into 1.2 ml of potassium phosphate buffer, pH 7.5, containing 0.5% Triton X-100, and lactate dehydrogenase activity was determined, using a kit (Sigma) according to the instructions of the manufacturer, as a measure of cell survival (Sapolsky, 1986; Packan and Sapolsky, 1990). Control plates that were not exposed to steroid or carbenoxolone were taken to represent 100% survival, and blanks represented 0% survival.

Statistics. Three to eight plates of cells were used for each data point. Data were assessed by ANOVA followed by Newman–Keuls *post hoc* test or Student's *t* tests, where appropriate. Significance was set at $p < 0.05$. Values are mean ± SEM.

RESULTS

11β-HSD in cultured hippocampal cells

Primary fetal hippocampal cell cultures showed clear 11β-HSD activity in cell homogenates. $NADP^+$-dependent 11β-dehydrogenase and NADPH-dependent 11β-reductase activity was clearly detectable after 1 hr incubation (Fig. 1). NAD^+-dependent activity was only just above basal enzyme activity (with no added cosubstrate; Fig. 1). PCR analysis showed clear expression of 11β-HSD1 mRNA, but no detectable expression of 11β-HSD2 mRNA (Fig. 2). Northern analysis showed only one hybridizing

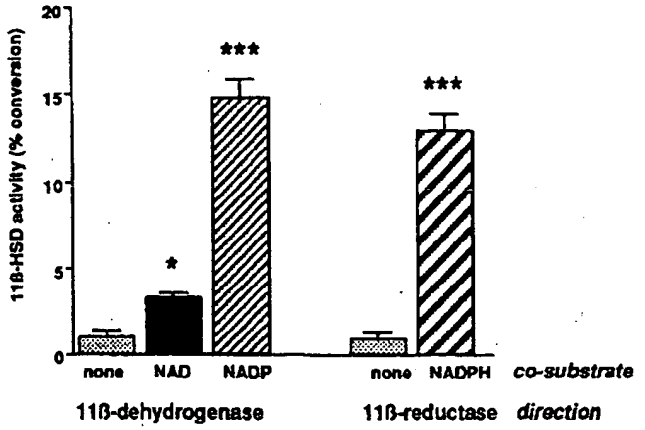

Figure 1. Cosubstrate dependence and reaction direction of 11β-HSD in homogenates of cultured primary hippocampal cells. Cosubstrates were added at 400 μM. Note the predominant $NADP^+$/NADPH-dependent reaction, typical of 11β-HSD1, and the obvious bidirectional activity in homogenates. $^*p < 0.05$ and $^{***}p < 0.0001$ compared with the appropriate control without exogenous cosubstrate.

species of 11β-HSD1 transcript, identical in size to the transcript in rat liver (data not shown), confirming previous studies of 11β-HSD1 transcripts in adult and fetal rat hippocampus *in vivo* (Moisan et al., 1990, 1992; Low et al., 1994b).

In contrast, predominant 11β-reduction was found (Fig. 3) in intact hippocampal cells, with clearly detectable conversion of inert 11-dehydrocorticosterone to corticosterone within 30 min of addition of steroid, and 78% conversion after 24 hr. This represented plateau activity because no further conversion occurred after 48 hr incubation with [^{3}H]A (data not shown). 11β-Dehydrogenase activity only became detectable after 8 hr incubation with [^{3}H]B (4 ± 1% conversion) and reached a mere 6.5 ± 1% conversion at 24 hr (Fig. 3). Pretreatment of cultures with the 11β-HSD inhibitor carbenoxolone (10^{-6} M) almost completely inhibited enzyme activity in intact hippocampal cells in

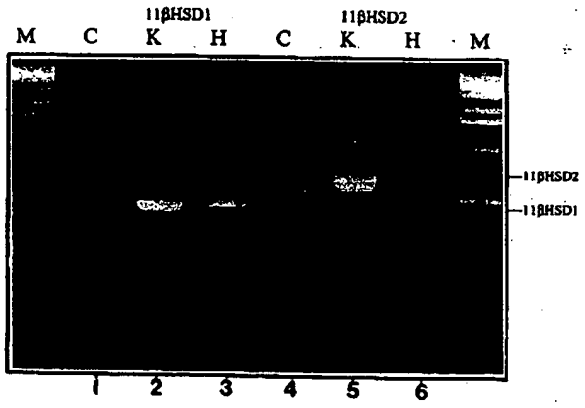

Figure 2. Presence of 11β-HSD1 mRNA in cultured primary hippocampal cells. Total RNA from 11-d-old cultures was subjected to reverse transcription followed by PCR using primers specific for 11β-HSD1 and 11β-HSD2. *Lane 1*, Negative control (*C*); *lane 2*, positive control kidney RNA (*K*); *lane 3*, hippocampal cell culture RNA (*H*); *lane 4*, negative control (*C*); *lane 5*, positive control kidney RNA (*K*); and *lane 6*, hippocampal cell culture RNA (*H*). *M* indicates marker lanes. Reactions in *lanes 1, 2,* and *3* contained 11β-HSD1-specific primers, and *lanes 4, 5,* and *6* contained 11β-HSD2-specific primers. Note the presence of a band in *lane 3* corresponding to hippocampal 11β-HSD1. The band in *lanes 5* and *6* migrating at ~0.9 kb corresponds to DNA-generated PCR product (see Results).

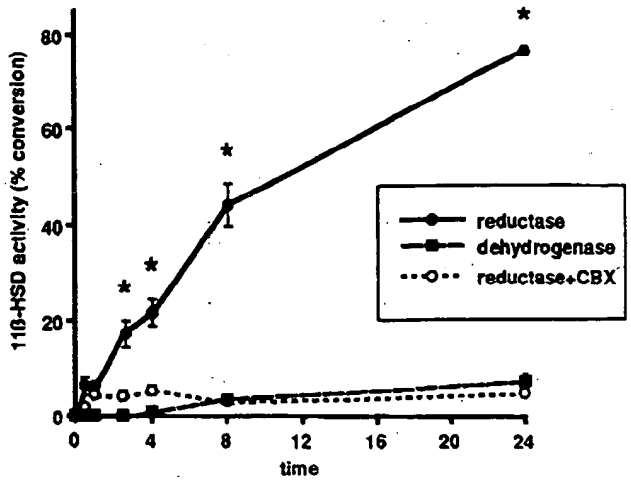

Figure 3. Reaction direction of 11β-HSD in intact primary hippocampal cells *in vitro*. [^{3}H]Corticosterone or [^{3}H]11-dehydrocorticosterone was added to the culture medium, and [^{3}H]corticosteroids in the medium were assayed at intervals. Note the predominant 11β-reduction in intact hippocampal cells. Carbenoxolone (CBX; 10^{-6} M) inhibits the reaction. $^*p < 0.01$ for 11β-reductase activity compared with either carbenoxolone-inhibited 11β reductase or 11β-dehydrogenase at the same time point.

both 11β-reductase (Fig. 3) and 11β-dehydrogenase (data not shown) directions.

Glucocorticoid modulation of hippocampal cell 11β-HSD activity

Treatment of primary hippocampal cell cultures with dexamethasone (10^{-7} M) for 72 hr increased 11β-HSD activity in cell homogenates (102% increase in 11β-dehydrogenase, 72% increase in 11β-reductase). In intact cells, this was exclusively an increase in 11β-reductase activity (by 43%), with no alteration in dehydrogenation detected (Fig. 4).

Effect of 11β-HSD on hippocampal cell survival in the presence of kainic acid

Preliminary experiments showed that both 10^{-7} and 10^{-5} M corticosterone potentiated kainic acid-mediated neurotoxicity, but this was considerably more apparent with the higher dose (data not shown). We therefore used the 10^{-5} M dose to study the effect of 11β-HSD in these cells. In the presence of corticosterone, kainic acid exerted significantly greater neurotoxicity than did kainic acid alone (Fig. 5), confirming previous studies (Sapolsky, 1986; Packan and Sapolsky, 1990). Carbenoxolone (10^{-6} M) did not alter the effect of corticosterone (10^{-5} M) on hippocampal cell loss in the presence of corticosterone, nor did 10^{-6} M carbenoxolone alone affect cell loss in the face of kainic acid stimulation (Fig. 5). 11-Dehydrocorticosterone (10^{-5} M) also potentiated kainic acid neurotoxicity and was at least as potent in this action as corticosterone (Fig. 5). However, inhibition of 11β-HSD with carbenoxolone (10^{-6} M) abolished the cytotoxic potentiating effects of 11-dehydrocorticosterone in hippocampal cultures.

DISCUSSION

These studies clearly demonstrate 11β-HSD activity in primary hippocampal cell cultures. A majority of the cultured cells were neurons, and it seems likely that the activity, taken together with previous immunocytochemical and *in situ* hybridization data showing a predominant neuronal localization of 11β-HSD in several brain regions *in vivo* (Moisan et al., 1990; Sakai et al., 1992), reflects 11β-HSD largely in neurons in the fetal hippocam-

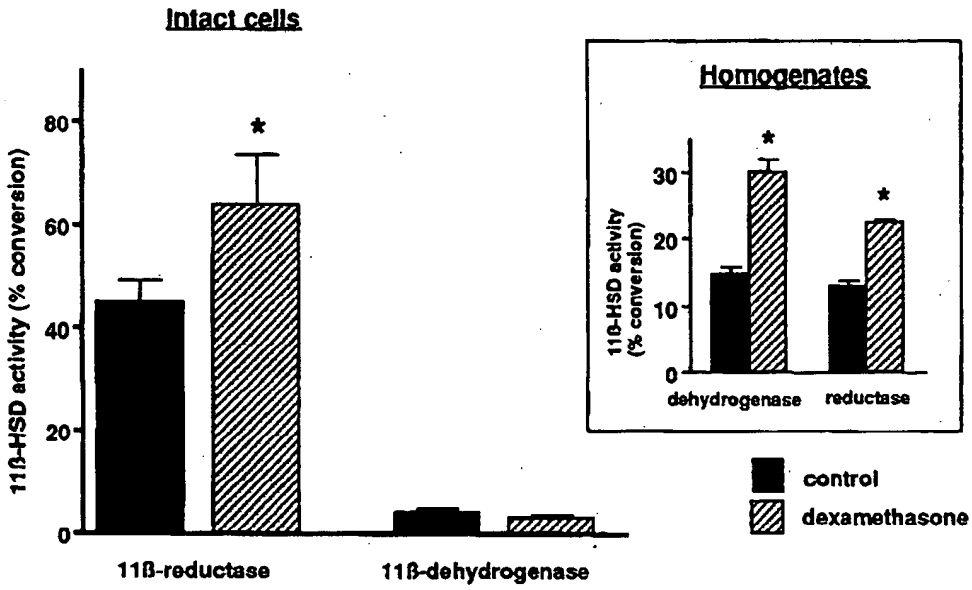

Figure 4. Effect of dexamethasone (10^{-7} M for 72 hr) on 11β-HSD activity in intact primary hippocampal cells and cell homogenates (*inset*). Note that dexamethasone induces hippocampal cell 11β-HSD activity *in vitro*, but the reaction is 11β-reduction in intact cells. $^{*}p < 0.05$ compared with control.

pal cultures. 11β-HSD activity was detectable in culture for more than 8 d, suggesting that it is stable under the conditions that were used. In intact hippocampal cells, 11β-reduction was clearly the predominant reaction direction. Dehydrogenation was barely detectable in intact hippocampal cells, and at the 8 hr time point at which 11β-dehydrogenation became detectable and 11β-reduction had not reached plateau values, the ratio of reduction to dehydrogenation was 11.5:1. Few previous studies have addressed the reaction direction of 11β-HSD in brain *in vivo*. Those reported have used peripheral injection of radiolabeled glucocorticoids and have examined the concentrations of 11-hydroxy to 11-keto products in target tissues (Burton and Tufnell, 1967). Uptake into the brain is low using such approaches, and in any event the data are complicated by peripheral conversion of steroids before entry into the brain, which largely reflects the balance of 11β-reduction in the liver and 11β-dehydrogenation in the kidney. No studies have examined 11β-reduction in brain *in vivo*, and indeed it is difficult to envisage satisfactory experimental protocols to overcome peripheral interconversion of corticoids without their direct infusion into the central nervous system.

In contrast, in homogenates of hippocampal cells, 11β-dehydrogenase was readily detectable and indeed exceeded 11β-reductase activity. This confirms studies in homogenates of hippocampus *in vivo*, which show both dehydrogenation and reduction reactions (Lakshmi et al., 1991; Seckl et al., 1993). The basis for the discrepancy between predominant reduction in intact cells and dehydrogenation in homogenates of these cells is unclear but not unique to the hippocampus. Thus, transfection of COS7 cells with an expression plasmid encoding 11β-HSD1 produces exclusively 11β-reductase activity in intact cells, but potent dehydrogenation is revealed when the transfected cells are homogenized (Low et al., 1994a). Reaction direction has been suggested to be determined by the glycosylation status of the enzyme or the tissue cosubstrate condition (Agarwal et al., 1990; Monder and White, 1993). Variations in glycosylation cannot explain near-exclusive reduction in intact hippocampal cells when dehydrogenation predominates immediately after these cells are homogenized. Moreover, any variations in $NADP^+$/NADPH ratios are unlikely to be sufficient to account for the dramatic change in reaction direction observed, and even gross changes in these ratios in intact cells have little effect on reaction direction of 11β-HSD1, at least in the liver (Jamieson et al., 1995). Thus, it seems more likely that the subcellular context of the enzyme determines the reaction direction, with homogenization disrupting this. Whether or not the reductase component is unstable in homogenates, it is clear from these data that dehydrogenation is revealed by cellular disruption, but it is at most a minor reaction in intact hippocampal cells and, by implication, in the hippocampus *in vivo*. The lack of 11β-dehydrogenation in intact hippocampal cell cultures also concurs with the nonselectivity of hippocampal MRs *in vivo* (de Kloet et al., 1975, 1991; Reul and de Kloet, 1985; McEwen et al., 1986a). Whether 11β-HSD2 (an exclusive dehydrogenase) is present in the hippocampus is still debatable. Northern analysis shows no expression of 11β-HSD2 mRNA in whole (human) brain

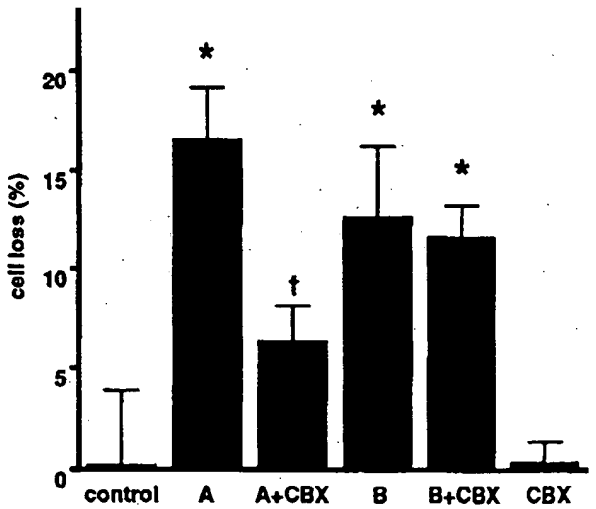

Figure 5. Effect of pretreatment with corticosterone (*B*; 10^{-5} M) and 11-dehydrocorticosterone (*A*; 10^{-5} M) with or without carbenoxolone (*CBX*; 10^{-6} M) on hippocampal cell loss after exposure to kainic acid (10^{-5} M). Carbenoxolone alone has no effect on cells and does not alter the neurotoxic potentiation of corticosterone (*B* vs *B+CBX*). However, 11-dehydrocorticosterone (*A*) toxicity is prevented by carbenoxolone (*A* vs *A+CBX*), indicating that hippocampal cell 11β-HSD acts as a functional 11β-reductase in this model. $^{*}p < 0.05$ compared with control; $^{\dagger}p < 0.05$ compared with *A* alone.

(Albiston et al., 1994), and *in situ* hybridization studies have failed to find 11β-HSD2 mRNA in the adult rat hippocampus (Roland et al., 1995). In contrast, a recent PCR-based study showed 11β-HSD2 transcripts in adult hippocampus (Zhou et al., 1995). Using a similar PCR approach, however, we could not detect 11β-HSD2 mRNA in primary hippocampal cell cultures and, importantly, found little NAD^+-dependent bioactivity. Thus, hippocampal expression of this MR-protective 11β-HSD isoform is probably negligible, at least in adult and late fetal life.

Previous studies have shown that chronic glucocorticoid (dexamethasone) excess or stress increase 11β-HSD1 mRNA expression and enzyme bioactivity (measured as dehydrogenation in homogenates) in the rat hippocampus (Low et al., 1994b). Because treatment of primary hippocampal cultures with dexamethasone increased 11β-HSD activity, it is probable that this effect of glucocorticoids is mediated directly on hippocampal neurons, and there is a putative glucocorticoid response element in the promoter of the rat 11β-HSD1 gene (Moisan et al., 1992). Moreover, preliminary data from transfection experiments with plasmids in which 11β-HSD1 promoter DNA is fused to a reporter gene suggest that a functional glucocorticoid response element lies within 3700 base pairs of the transcription start site (K. E. Chapman, M. Voice, R. Wallace, V. Lyons, and J. R. Seckl, unpublished observations). Although it has been proposed that induction of 11β-HSD in the hippocampus may protect vulnerable neurons from the deleterious consequences of chronic glucocorticoid excess (Monder, 1991; Low et al., 1994b), the activity induced by dexamethasone was exclusively 11β-reductase, a finding incompatible with this notion. Thus, hippocampal 11β-HSD may potentiate rather than attenuate the neurotoxic effects of chronic glucocorticoid overexposure.

To examine this further, the effects of enzyme inhibition in both reductase and dehydrogenase directions was determined by using carbenoxolone. Potentiation of kainic acid neurotoxicity with 10^{-7} and 10^{-5} M corticosterone was found in primary hippocampal cell cultures, confirming previous studies (Sapolsky, 1986; Packan and Sapolsky, 1990). Although supraphysiological levels of steroids were used *in vitro*, physiological concentrations (10^{-7} M) are effective in potentiating kainic acid neurotoxicity in this system (Packan and Sapolsky, 1990; and these data), although the changes were small during the short time course used here. *In vivo*, corticosteroid effects take many days to become manifest (Sapolsky et al., 1985), and we have used higher concentrations to amplify effects in cell cultures. That the effect of corticosterone was unaltered by carbenoxolone in a dose that inhibited both reaction directions but had no direct effect on cell survival supports the relative lack of importance of 11β-dehydrogenation in these cells. 11-Dehydrocorticosterone, which has 10^4-fold lower affinity for receptors than does corticosterone (Ulmann et al., 1975; Armanini et al., 1983), was at least as effective as corticosterone in potentiating kainic acid toxicity. The functionality of 11β-reductase was clear, inasmuch as 11-dehydrocorticosterone was ineffective when 11β-HSD was inhibited by carbenoxolone. These data support the predominant 11β-reductase action of hippocampal 11β-HSD.

The reasons for an enzyme regenerating active glucocorticoids in the hippocampus are obscure. Hippocampal cells are exquisitely sensitive to glucocorticoid concentrations, with granular neuronal dysfunction and possibly death in the dentate gyrus after adrenalectomy (Sloviter et al., 1989; Gould et al., 1990) and pyramidal neuronal loss in the cornu ammonis with glucocorticoid excess (Landfield et al., 1978; Sapolsky et al., 1985, 1986; Sapolsky, 1985, 1992; Landfield and Eldridge, 1991). In the rat, corticosterone levels are very low during the day but show a pronounced diurnal increase in the evening. The biological importance of this rhythm is unclear, but the maintenance of many constitutive cellular functions may require more than the minimal levels of circulating corticosterone that pertain during the majority of the day. Thus, local 11β-reduction may increase corticosterone levels in specific tissues and, hence, 11-dehydrocorticosterone may form a circulating reservoir of inert corticosteroid for cell-specific activation (the apparent absence of 11β-HSD2 in the hippocampus suggests that 11-dehydrocorticosterone comes from the periphery rather than from local cellular production). Certainly in humans, cortisone (the equivalent of 11-dehydrocorticosterone) shows near-constant levels throughout the 24 hr period. Moreover, plasma concentrations of cortisone (which circulates largely unbound at ~100 nmol/l) approximate or even exceed "free" cortisol levels, providing plentiful substrate for 11β-reductase (Walker et al., 1992). Similarly, levels of 11-dehydrocorticosterone at ~50 nmol/l are found in rat plasma (R. Best and J. R. Seckl, unpublished observations), concentrations well in excess of "free" corticosterone levels during the diurnal nadir. Such cell-specific activation of an inert circulating form is not unique to glucocorticoids and may be analogous to the activation of thyroxine to tri-iodothyronine by 5′-monodeiodinase and testosterone to dihydrotestosterone by 5α-reductase in other tissues.

Why the activity of hippocampal 11β-reductase should increase with chronically elevated glucocorticoids also is unclear, because this seems to increase the neuron-jeopardizing effects of glucocorticoid excess. Perhaps the short- and medium-term metabolic and functional benefits of maximizing glucocorticoid exposure during stress outweigh any long-term detriments, particularly because potent negative feedback effects would be expected to rapidly attenuate glucocorticoid levels under most physiological circumstances. Further determination of the importance of hippocampal 11β-HSD activity will be assisted by the development of selective 11β-reductase inhibitors or transgenic animals lacking 11β-HSD1. Nevertheless, it is intriguing to speculate that measures to attenuate hippocampal 11β-reductase may reduce neuronal vulnerability to glucocorticoid toxicity in a target-specific manner, analogous to the effects of long-term maintenance of more generalized low glucocorticoid levels by adrenalectomy (Landfield et al., 1978) or increasing sensitivity to glucocorticoid negative feedback (Sapolsky et al., 1984; Meaney et al., 1988, 1993; Seckl and Olsson, 1995).

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VITAMINS AND HORMONES, VOL. 47

11β-Hydroxysteroid Dehydrogenase

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I. Historical Origins

It was experimentally established in 1856 (Brown-Sequard, 1856) that adrenalectomy in animals is fatal, but not until 1927 was it shown

by Rogoff and Stewart (1927) that adrenal extracts could maintain adrenalectomized animals. By 1937, Reichstein in Basel, Switzerland, and Kendall in Rochester, Minnesota, had demonstrated that the active substances in adrenal cortical extracts were steroids. In that year Reichstein and his colleagues synthesized 11-deoxycorticosterone (Steiger and Reichstein, 1937), which, although it was not a quantitatively important secretory product of the adrenal cortex, was used for many years to treat Addisonian patients with some, but by no means total, success. A consensus soon emerged that the therapeutically active adrenal steroid contained oxygen at C-11 (Kendall, 1941; Ingle, 1940; Thorn, 1944; Olson *et al.*, 1944). What complicated discussion of the identity of the hormone of the adrenal cortex was the large number of steroids extracted from slaughterhouse tissue (Fieser and Fieser, 1959). Many of these were 11-oxygenated and were thus candidates for the active steroid. Studies with isolated, perfused adrenals (Reichstein and Shoppee, 1943), analysis of urine from normal individuals (Conn *et al.*, 1951) or patients with Cushing's disease (Mason, 1950; Sprague *et al.*, 1951), and adrenal vein blood (Reich *et al.*, 1950; Nelson *et al.*, 1951; Bush, 1953) led to the conclusion that cortisol (F) and corticosterone (B) are the primary secretory products of the adrenal gland. There was evidence that adrenal enzymes were capable of oxidizing the 11-hydroxy group of corticosterone and cortisol to an 11-keto group (Hechter *et al.*, 1951; Burstein *et al.*, 1953; Fazekas *et al.*, 1970), and that patients treated with cortisone (E) excreted some F (Mason, 1950; Sprague *et al.*, 1951; Burton *et al.*, 1953; Miller and Axelrod, 1953). Although the interconversion of the 11-oxygenated pairs, F and E, and B and 11-dehydrocorticosterone (A) could be readily demonstrated, their biological relationships to each other were not obvious. It was suggested that both cortisol and cortisone were converted to the true active steroid (Hechter *et al.*, 1953). However, cortisone was ineffective when injected into arthritic joints, in contrast with the pronounced antiarthritic effects of the orally administered steroid (Hollander *et al.*, 1951; Zacco *et al.*, 1954). The conclusion that cortisone is biologically inactive and must be converted to its physiologically active form, cortisol, by reduction of the 11-oxo group was supported by other clinical observations (Boland, 1952; Dixon and Bywaters, 1953; Cope and Hurlock, 1954), and by studies with experimental animals (Eisenstein, 1952; Fish *et al.*, 1953; Burton *et al.*, 1953; Caspi *et al.*, 1953; Amelung *et al.*, 1953a; Dobriner, 1951; Savard *et al.*, 1953). In 1953, sufficient quantities of cortisol became available to permit its metabolism to be studied (Fieser and Fieser, 1959). Oral administration of cortisol acetate to human subjects resulted in the

excretion of 11-oxo C_{21} and C_{19} steroids (Burstein *et al.*, 1953). An enzyme responsible for catalyzing the oxidation of cortisol to cortisone was found in rat liver (Amelung *et al.*, 1953a,b) and named "11β-hydroxy dehydrogenase" (Hubener *et al.*, 1956). It is now known as 11β-hydroxysteroid dehydrogenase (11-HSD). Figure 1 illustrates the transformations catalyzed by this enzyme.

II. Distribution, Properties, and Behavior of 11-HSD

A. Tissue Distribution

Catalysis of 11-oxidation and 11-oxoreduction is not uniformly distributed among tissues. In liver, 11-oxoreduction is the dominant activity; in most other tissues, it is 11β-hydroxy oxidation. Whether this behavior is due to the expression of separate enzymes or to the tissue-specific behavior of a unique 11β-hydroxysteroid dehydrogenase was a question first posed 35 years ago (Bush, 1956, 1959; Bush and Mahesh, 1959a). Most investigators have interpreted the results of their studies on steroid metabolism at position 11 in terms of a single enzyme, designated by the Nomenclature Committee of the International Union of Biochemistry as EC 1.1.1.146 (11β-hydroxysteroid:NADP$^+$ 11-oxidoreductase) (Webb, 1984). Within this context, there have been suggestions of multiple enzyme forms, based on the fact that the char-

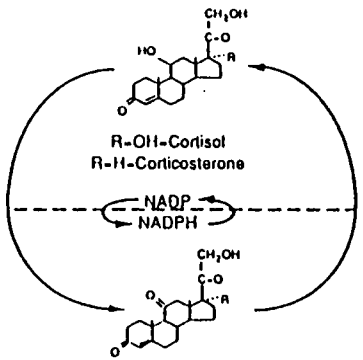

FIG. 1. Transformations catalyzed by 11β-hydroxysteroid dehydrogenase.

acteristics of the enzyme in different tissues varied over a range that was too wide to be accounted for by error or interlaboratory variability.

Table I summarizes our current knowledge of the tissue distribution of 11-HSD. For convenience, activity reported to occur in the oxidative direction is called corticosteroid 11β-dehydrogenase (11-DH), and reductive activity is called corticosteroid 11-oxoreductase (11-OR). From the available literature, it is difficult to evaluate whether 11-DH and 11-OR activities are both present in a particular organ (Fish *et al.*,

TABLE I
DISTRIBUTION OF 11-HSD IN MAMMALIAN TISSUES*

Tissue	11β-Dehydrogenase	11-Oxoreductase
Liver	H, D, M, Rb, G, Rt	M, Rb, G, Rt, C
Kidney	H, D, M, Rb, G, Rt	—
Lung	D, M, Rb, G, Rt	H, M, Rb, Rt
Testis	H, Rb, Rt, M	Rt
Brain	D, M, Rt, B	R
Spleen	D, M, G	M
Adrenal cortex	H, D, Rb, Rt, Sh, C, MV	MV
Diaphragm	D, Rt	—
Skeletal muscle	M, Rt, C	—
Blood vessels	Rb, Rt	—
Heart	Rt	—
Lymphocytes	M, Rt	Rt
Thymocytes	M, Rt	M, Rt
Small intestine	H, D, Rt	—
Colon	Rt	—
Placenta	H, Rt, B	H
Ovary	—	—
Uterus	H	H
Myometrium	—	—
Amniotic membrane	H	H
Decidua	H	H
Chorion	H	H
Adipose tissue	H	—
Salivary gland	Rt	—
Mammary gland	H	—
Skin	—	H
Gingival tissue	—	H

*H, human; D, dog; M, mouse; Rb, rabbit; G, guinea pig; Rt, rat; C, cattle; B, baboon; MV, meadow vole; Sh, sheep. The table cites positive identification of 11-HSD in the oxidative direction (11β-dehydrogenase) or in the reductase direction (11-oxoreductase) in the investigated species. Absence of measureable activity or no reported activity is indicated by a dash.

1953; Bush *et al.*, 1968; Koerner, 1969; Monder and Lakshmi, 1989a). Where reversibility has been reported, the results have not generally been consistent. The 11-HSD in human adipose tissue has been reported to catalyze only oxidation. Results with intestinal mucosa and skin (Murphy, 1981; Hsia and Hao, 1966; Hammami and Siiteri, 1990; Burton and Anderson, 1983) are in conflict. The reasons for the great differences between laboratories are not clear, but the following sources of variation are probably important; (*a*) the pH of measurement; (*b*) the relative stabilities of the dehydrogenase and oxoreductase activities (Lakshmi and Monder, 1985a); (*c*) incompletely expressed or "latent" enzyme (Lakshmi and Monder, 1985b); (*d*) the age of the animal, its sex and diet, and the possible presence of endogenous inhibitors or activators; (*e*) the developmental stage of the animal (Murphy, 1981); (*f*) substrate specificity (Koerner, 1969).

B. Physiological Functions

It has been suggested that 11-HSD protects cells against the toxic effects of excess corticosteroid (Dougherty *et al.*, 1961; Berliner, 1965). 11β-Dehydrogenase may be a component of a degradation pathway, strategically placed to inactivate corticosteroids prior to their exposure to receptor or to prevent the return of the steroid to receptor. The enzyme also serves a conservationist function, since the oxidized form of the steroid can be reduced by 11-oxoreductase to its active reduced form thus contributing to the circulating cortisol, and providing a buffer against the changes in blood level caused by paroxysmal secretion of the adrenal. The enzyme can thus integrate the availability of corticosteroids to target organs and their metabolism. An overview of these proposed interrelationships is presented in Fig. 2.

C. Enzymatic Properties

1. *Substrate Specificity*

In Tables IIa and IIb are listed all steroids for which oxidation or reduction catalyzed by 11β-hydroxysteroid dehydrogenase have been reported. Table IIc lists the steroids that were found to be neither oxidized nor reduced at C-11. Based on the data in Table II the qualitative effects of substituents on oxidoreduction, i.e., whether steroids carrying the indicated functional group are substrates for 11-HSD, are summarized in Table III.

From the data summarized in Tables II and III it is possible to deduce

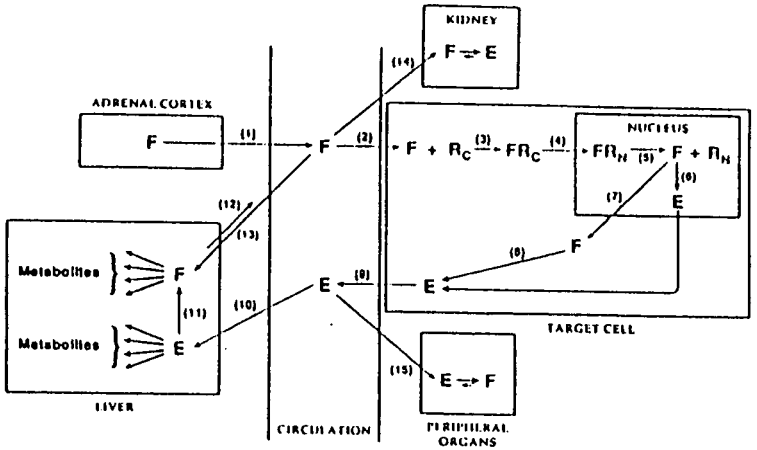

FIG. 2. An overview of the proposed role of 11β-hydroxysteroid dehydrogenase in modulating corticosteroid function. In the model, 11β-hydroxysteroid function and metabolism are illustrated with cortisol (F), and its 11-oxo derivative, cortisone (E). F, synthesized in the adrenal cortex, is transported through the circulation (1) to its target cell (2). The steroid binds to the cytosolic receptor (R_C) (3), which is transported to the nucleus (4), or within the nucleus to the resident receptor (R_N). At an as yet undetermined point in the cell response process the steroid released (5) from the R_N is oxidized to E by nuclear 11-HSD (6), or it leaves the nucleus (7) and is oxidized by microsomal 11-HSD (8). The F thus formed is transported in the circulation (9) to the liver (10) where it is metabolized to inactive end products, or converted to cortisol by 11-oxoreductase (11) which is metabolized or returned to the circulation (12). Circulating cortisol contributes to the liver cortisol pool, as well (13). The kidney is a major contributor to cortisol oxidation (14). Cortisone may be converted to active steroid by peripheral tissues (15) as well as liver; however, evidence for this regenerative pathway is scanty.

how structural changes in steroids bring about corresponding changes in bioactivity. There is considerable evidence that any substituent that inhibits oxidation of the 11β-hydroxy group can enhance corticosteroid potency even if the structural modification does not have an enhancing effect on some other parameter, such as receptor affinity. Bush *et al.* (1968) and Koerner (1969) have analyzed the effects of substituents on oxidation or reduction of 11-oxygenated steroids. From their data, the following conclusions may be drawn. (*a*) A flat A/B junction (5α) is essential for binding to the active site, whereas a buckled A/B junction (5β) prevents binding; (*b*) bulky groups that obstruct the α-surface (2α-

TABLE II
STEROID SUBSTRATES OF 11β-HYDROXYSTEROID DEHYDROGENASE

(a) 11-OH → 11-oxo	
Cortisol	Osinski (1960); Koerner (1969)
Corticosterone	Osinski (1960); Koerner (1969)
11β,17,20β,21-Tetrahydroxypregn-4-en-3-one	Bradlow *et al.* (1968)
11β-Hydroxypregn-4-en-20-one	Koerner (1969); Bush *et al.* (1968)
3α,11β,17,21-Tetrahydroxy-5α-pregnan-20-one	Bush and Maheah (1959a); Koerner (1969)
11β,17α,20β,21-Tetrahydroxypregn-4-en-3-one	Koerner (1969)
11β,17α,21-Trihydroxy-5α-pregnan-3,20-dione	Koerner (1969)
11β-Hydroxyandrost-4-en-3,17-dione	Koerner (1969)
11β,17,21-Trihydroxy-5α-pregnan-3,20-dione	Koerner (1969)
3β,11β,17,21-Tetrahydroxy-5α-pregnan-20-one	Koerner (1969)
3β,11β,17,21-Tetrahydroxy-pregn-5-en-20-one	Koerner (1969)
11β,17,21-Trihydroxypregn-1,4-diene-3,20-dione	Koerner (1969)
11β,17,20α,21-Tetrahydroxypregn-4-en-3-one	Koerner (1969)
11β,17-Dihydroxypregn-4-ene-3,20-dione	Koerner (1969)
16α-Methyl-cortisol	Bush *et al.* (1968)
16β-Methyl-cortisol	Bush *et al.* (1968)
3α,11β,17,21-Tetrahydroxy-5α-pregnan-20-one-3-acetate	Koerner (1969)
(b) 11-oxo → 11β-OH	
Cortisone	Fish *et al.* (1953); Burton *et al.* (1953)
21-Hydroxypregn-4-en-3,11,20-trione	Mason (1950)
Androst-4-ene-3,11,17-trione	Hubener *et al.* (1956); Bush *et al.* (1968)
Pregn-4-ene-3,11,20-trione	Hubener *et al.* (1956); Bush *et al.* (1968)
17α,20β,21-Trihydroxypregn-4-en-3,11-dione	Koerner (1969); Hubener *et al.* (1956)
17,21-Dihydroxy-pregn-1,4-diene-3,11,20-trione	Bush *et al.* (1968)
17,21-Dihydroxy-5α-pregnan-3,11,20-trione	Bush *et al.* (1968)
9α-Fluorocortisone	Bush *et al.* (1968)
9α-Fluoro-11-oxo-progesterone	Bush *et al.* (1968)
12α-Fluoro-11-oxo-progesterone	Bush *et al.* (1968)
12α-Bromo-11-oxo-progesterone	Bush *et al.* (1968)
3α-Hydroxy-5α-androstane-11,17-dione	Bush *et al.* (1968)
9α-Fluoro-androst-4-en-3,11,17-trione	Bush *et al.* (1968)
9α-Chlorocortisone	Bush *et al.* (1968)
9α-Chloro-androst-4-ene-3,11,17-trione	Bush *et al.* (1968)
12α-Bromo-11-dehydrocorticosterone	Bush *et al.* (1968)
(c) Unreactive steroids	
Tetrahydrocortisol	Hubener *et al.* (1956); Bradlow *et al.* (1968)

(continued)

TABLE II (*Continued*)

Tetrahydrocortisone	Hubener *et al.* (1956)
3α-Hydroxy-5β-androstan-11,17-dione	Hubener *et al.* (1956); Bush and Mahesh (1959b)
17α,20β,21-Trihydroxypregn-4-ene-3,11-dione	Bradlow *et al.* (1968)
11β,17α,21-Trihydroxy-5β-pregnan-3-one	Bush *et al.* (1968); Koerner (1969)
2α-Methylcortisone	Bush and Mahesh (1959b)
2α-Methylcortisol	Bush and Mahesh (1959b)
3α,17α,20α,21-Tetrahydroxy-5β-pregnan-11-one	Huebener *et al.* (1956)
3α,17α,20β,21-Tetrahydroxy-5β-pregnan-11-one	Huebener *et al.* (1956)
11β-Hydroxyestrone	Koerner (1969)
12α-Bromo-11-dehydrocorticosterone	Bush *et al.* (1968)
12α-Methyl-11-oxoprogesterone	Bush *et al.* (1968)
9α-Fluorocortisol	Bush *et al.* (1968)
12α-Bromo-11β-hydroxyprogesterone	Bush *et al.* (1968)
12α-Fluorocorticosterone	Bush *et al.* (1968)
12α-Bromocorticosterone	Bush *et al.* (1968)
2α-Methyl-9α-fluorocortisol	Bush *et al.* (1968)
2α-Methyl-9α-fluoro-11β-hydroxyprogesterone	Bush *et al.* (1968)
11β,17,21-Trihydroxy-16α-methyl-pregn-1,4-diene-3,20-dione	Bush *et al.* (1968)
9α-Fluoro-11β,16α,17α,21-tetrahydroxypregn-1,4-diene-3,20-dione	Bush *et al.* (1968)
2α-Methyl-androst-4-ene-3,11,17-trione	Bush *et al.* (1968)
Cortisol-21-sulfate	Koerner (1969)
Cortisol-21-phosphate	Koerner (1969)
Cortisol-21-acetate	Koerner (1969)
Cortisol-21-hemisuccinate	Koerner (1969)
3α-Hydroxy-5β-androstane-11,17-dione	Bush *et al.* (1968)

methyl) inhibit binding; (c) aromatic A ring is forbidden; (*d*) effects of halogens are more likely to be the consequence of their inductive effects than their steric effects; (*e*) steroids with bulky substituents (acetyl, phosphate) at C-21 are not substrates. Structural studies lead to the conclusion that the steroid α-surface binds to the enzyme, and that hydrogen transfer occurs from the 11α-position. Consequently, variations in the velocities of nonhalogenated steroids can be attributed to steric factors.

The inability of 2α-methyl steroids to be oxidized or reduced at C-11 played an important historical role in reinforcing the conclusion that cortisol, and not cortisone, was the active steroid hormone (Bush and Mahesh, 1959b). The metabolically active 2α-methyl-11β-hydroxysteroid could not be oxidized by 11-HSD and the 2α-methyl-11-oxosteroid was inactive as a glucocorticoid, supporting the importance

TABLE III
EFFECTS OF FUNCTIONAL GROUPS ON SUBSTRATE SPECIFICITY OF 11-HSD

Functional group	Oxidation[a]	Reduction[a]
1-one	+	+
2α-Methyl	−	−
3α-Hydroxy	+	NR
3β-Hydroxy	+	NR
Δ⁴-3-Oxo	+	+
5β	−	−
5α	+	+
9α-Fluoro	−	+
12α-Fluoro	−	+
16α-Methyl	+	NR
17α-Hydroxy	+	+
20-Hydroxy (α or β)	+	+
21-Methyl	+	+
21-Hydroxy	+	+

[a] +, steroid with indicated functional group is a substrate; −, steroid with indicated functional group is not a substrate. NR, not reported. In evaluating the effects of multiple substituents on substrate specificity, "−" precedes "+." Substituents for which only single examples exist are omitted here, and are listed in Table II.

of the 11β-hydroxy group in glucocorticoid function. These results also helped to disprove the hypothesis that steroids affect metabolism by directly participating as cofactors in transhydrogenation reactions (Williams-Ashman and Liao, 1964).

2. *Steroid Inhibitors*

The catalysis of 11-oxidation is inhibited by a number of structurally diverse steroids, including representatives of the C_{21} and C_{19} series. Inhibitors of reduction have also been shown to include C_{21} and C_{19} steroids, though fewer studies have been performed in this direction. Some C_{18}, C_{19}, and C_{21} steroids inhibit neither oxidation nor reduction. The steroids that have been investigated for their ability to inhibit 11-HSD are listed in Table IV. On the basis of the data, we conclude that inhibition of 11β-dehydrogenase is not caused by the following: 2α-CH_3, 5β-H, 6α-OH, 6β-OH, 12α-OH, 15α-OH, 16α-OH, 20α-OH, 11-oxo, 18-oxo, 16(17)-ene. Steroids devoid of oxygen at C-11 are generally not inhibitors, or inhibit oxidation poorly. The 11α-

TABLE IV
STEROID INHIBITORS OF 11β-HYDROXYSTEROID DEHYDROGENASE

(a) Oxidation (11-OH → 11-oxo)	
C_{21} and C_{19} steroids	
11α,17,21-Trihydroxy-pregn-4-en-3-one (11-epicortisol)	Burton (1966)
11α,17,21-Trihydroxy-pregn-1,4-diene-3-one (11-epiprednisolone)	Burton (1965)
11α-Hydroxypregn-4-en-3-one (11α-hydroxyprogesterone)	Burton (1966); Murphy and Vedady (1982)
17,21-Dihydroxypregn-4-ene-3,11-dione (cortisone)	Bernal *et al.* (1980); Murphy (1979b)
Cortisol 21-acetate	Bernal *et al.* (1980)
Progesterone	Bernal *et al.* (1980); Murphy and Vedady (1982)
1-Dehydro-16α-methyl-9α-fluorohydrocortisone (dexamethasone)	Bernal *et al.* (1980)
11β,17,21-Trihydroxypregn-1,4-dien-3-one (prednisolone)	Bernal *et al.* (1980); Murphy and Vedady (1982)
9α-Fluorocortisol	Bush *et al.* (1968)
3α,11β,17,21-Tetrahydroxy-5α-pregnan-20-one (allotetrahydrocortisol)	Deckx and DeMoor (1966)
3α,11β,17,20β,21-Pentahydroxy-5α-pregnane (allocortol)	Deckx and DeMoor (1966)
11β,17α,21-Trihydroxy-5α-pregnane-3,20-dione (allodehydrocortisol)	Deckx and DeMoor (1966)
11β-Hydroxytestosterone	Monder and Lakshmi (1989a)
11β-Hydroxyandrost-4-ene-3,17-dione	Deckx and DeMoor (1966); Monder and Lakshmi (1989a)
3α,11β,17β-Trihydroxyandrostane	Monder and Lakshmi (1989a)
3β-Hydroxyandrost-5-en-17-one	Deckx and DeMoor (1966)
11β,17β-Dihydroxy-5β-androstan-3-one	Monder and Lakshmi (1989a)
11β,17β-Dihydroxy-5α-androstan-3-one	Monder and Lakshmi (1989a)
(b) Reduction (11-oxo → 11-OH)	
C_{21} and C_{19} steroids	
11-Oxoprogesterone	Tordoy *et al.* (1975)
3α,17,21-Trihydroxy-5β-pregnan-3-20-dione (tetrahydrocortisone)	Bernal *et al.* (1980)
21-Hydroxy-pregn-4-ene-3,11,20-trione	Bernal *et al.* (1980)
Androst-4-ene-3,11,20-trione	Deckx and DeMoor (1966)
3β-Hydroxyandrost-5-en-17-one	Deckx and DeMoor (1966)

(continued)

TABLE IV *(Continued)*

(c) Do not Inhibit (11β-OH → 11-oxo)	
C_{21} steroids	
21-Hydroxypregn-4-ene-3,20-dione	Murphy and Vedady (1982)
17α,21-Dihydroxypregn-4-ene-3,11-dione	Murphy and Vedady (1982)
11β-Hydroxypregn-4-ene-3,20-dione-21-sulfate	Murphy and Vedady (1982)
11β,17α-Dihydroxypregn-4-ene-3,20-dione-21-sulfate	Murphy and Vedady (1982)
6α-Hydroxypregn-4-ene-3,20-dione	Murphy and Vedady (1982)
12α-Hydroxypregn-4-ene-3,20-dione	Murphy and Vedady (1982)
6β,11β,17α,21-Tetrahydroxypregn-4-ene-3,20-dione	Murphy and Vedady (1982)
11β,21-Dihydroxy-18-oxo-pregn-4-ene-3,20-dione	Murphy and Vedady (1982)
15α-Hydroxypregn-4-en-3-one	Murphy and Vedady (1982)
16α-Hydroxypregn-4-en-3-one	Murphy and Vedady (1982)
3,20-Dioxo-pregn-4,16-diene	Murphy and Vedady (1982)
3α,20α-Dihydroxy-5β-pregnane	Murphy and Vedady (1982)
9α-Fluoro-11β,17α,21-trihydroxy-16β-methylpregn-1,4-diene-3,20-Dione	Murphy and Vedady (1982)
Tetrahydrocortisol	Bernal *et al.* (1980); Deckx and DeMoor (1966)
3α,11β,17,20α,21-Pentahydroxy-5β-pregnane (α-cortol)	Deckx and DeMoor (1966)
3α,11β,17,20β,21-Pentahydroxy-5β-pregnane (β-cortol)	Deckx and DeMoor (1966)
Tetrahydrocortisone	Bernal *et al.* (1980); Deckx and DeMoor (1966)
2α-Methylcortisol	Bush *et al.* (1968)
C_{19} steroids	
3β,11β-Dihydroxy-5α-androstan-17-one	Murphy and Vedady (1982)
3α,11β-Dihydroxy-5α-androstan-17-one	Murphy and Vedady (1982)
3β,11β-Dihydroxy-5β-androstan-17-one	Murphy and Vedady (1982)
3β,11β,16α-Trihydroxyandrost-5-en-17-one	Murphy and Vedady (1982)
3α,11β-Dihydroxy-5β-androstan-17-one	Monder and Lakshmi (1989a); Murphy and Vedady (1982)
3β-Hydroxy-androst-5-en-17-one-3-sulfate	Murphy and Vedady (1982)
Testosterone	Bernal *et al.* (1980)
5α-Dihydrotestosterone	Bernal *et al.* (1980)
3α-Hydroxy-5β-androstan-17	Deckx and DeMoor (1966)
3α-Hydroxy-5α-androstan-17-one	Deckx and DeMoor (1966)
Androstenedione	Deckx and DeMoor (1966)
Androst-4-ene-3,11,17-trione	Deckx and DeMoor (1966)
Dihydroepiandrosterone	Pepe and Albrecht (1984a)
11β-Hydroxy-5β-androstane	Monder and Lakshmi (1989a)
3α,11β-Dihydroxyandrosten-17-one	Monder and Lakshmi (1989a)

(continued)

TABLE IV *(Continued)*

C_{18}	
Estradiol	Bernal *et al.* (1980); Abramovitz *et al.* (1984)
Estriol	Bernal *et al.* (1980); Abramovitz *et al.* (1984)
Estrone	Abramovitz *et al.* (1984)
(d) Do not inhibit (11-oxo → 11-OH)	
C_{21}	
2α-Methylcortisone	Bush *et al.* (1968)
Cortisol	Bush *et al.* (1968)
20β-Cortol	Bush *et al.* (1968)
20α-Cortol	Bush *et al.* (1968)
3α,11β,17,20β-21-Pentahydroxy-5α-pregnan-20-one (allocortol)	Bush *et al.* (1968)
Cortolone	Bush *et al.* (1968)
C_{19}	
Androst-4-ene-3,17-dione	Bush *et al.* (1968)
3α-Hydroxy-5α-androstan-17-one	Bush *et al.* (1968)
3α-Hydroxy-5β-androstan-17-one	Bush *et al.* (1968)

hydroxysteroids are probably structural analogs and are competitive inhibitors (Burton, 1965; Bernal *et al.*, 1980; Murphy and Vedady, 1981). Although this may also be true for 11β-hydroxysteroids, it is not yet determined which are site-specific structural analogs and which are competitive substrates.

A second important binding site may be the side chain, which requires small bulk at C-21 (—CH_3, —CH_2 OH). Introduction of a bulky or charged group at C-21 may diminish the ability of the steroid to act as a substrate, but not as an inhibitor (compare 11β-hydroxypregn-4-ene-3,20-dione and 11β-hydroxypregn-4-ene-3,20-dione-21-sulfate) (Murphy, 1982). Cortisol-21-acetate is a potent inhibitor of decidual dehydrogenase (Bernal *et al.*, 1980), yet is not a substrate (Koerner, 1969).

Since 11β-hydroxy-9α-fluoro compounds are not oxidized by 11β-hydroxysteroid dehydrogenase, inhibition of cortisol oxidation by 9α-fluorocortisol and dexamethasone is due to displacement of the substrate at the active site by homologs in which a negative inductive effect of the halogen stabilizes the 11β-hydroxy group (Bush *et al.*, 1968; Bush and Mahesh, 1959a). Prednisolone is oxidized by 11-HSD (Koerner, 1969) and the reported inhibitory effect (Bernal *et al.*, 1980) is probably due to substrate competition. A similar explanation is applicable to inhibition of cortisol oxidation by corticosterone (Bernal *et al.*, 1980), which is a better substrate for 11-HSD (Engel *et al.*, 1955; Osinski, 1960; Koerner, 1969) than the former.

There are few studies on steroid inhibition of 11-oxoreduction. Most steroids tested (Table IVd) did not affect 11-oxoreductase. Of several that did, none could be shown to have functional groups specially associated with obligatory inhibition. Reduction of an inhibiting steroid at C-20 (tetrahydrocortisone → cortolone) eliminated its inhibitory effect, suggesting a possible orienting role of the side chain. However, since androgens were inhibitors of 11-oxoreductase, the side chain is not essential for binding to the reductase.

The magnitude of the inhibitory effects of steroid analogs differs between tissues. This is illustrated by the data of Bernal *et al.* (1980), who compared the effects of a variety of steroids on placental and decidual microsomes in the oxidative direction. They observed that testosterone, 5α-dihydrotestosterone, and tetrahydrocortisol inhibited the decidual enzyme, but not the placental enzyme. Perhaps the two organs contain distinct species of 11-HSD.

3. *Subcellular Localization*

11β-Hydroxysteroid dehydrogenase has been found in the microsomal fraction of liver (Ghraf *et al.*, 1975a; Hurlock and Talalay, 1959; Koerner, 1969; Bush *et al.*, 1968), kidney (Mahesh and Ulrich, 1960; Ghraf *et al.*, 1975a; Kobayashi *et al.*, 1987), gonads (Ghraf *et al.*, 1975a), placenta (Bernal *et al.*, 1980), spleen (Deckx and DeMoor, 1966), and lung (Nicholas and Lugg, 1982). Cytosol (post-100,000*g* supernatant) and mitochondria were devoid of activity, or when activity was present in these fractions, it was due to contamination with microsomal or nuclear debris. Several investigators have presented evidence for 11-HSD activity in nuclei. Hierholzer and co-workers (Kobayashi *et al.*, 1987; Schulz *et al.*, 1987; Hierholzer *et al.*, 1990a) confirmed the observation of Mahesh and Ulrich (1960) that kidney nuclei contain significant levels of enzyme activity. The K_m values in the experiments of Kobayashi *et al.* (1987) were 2.2×10^{-7} *M* (microsomes) and 2.7×10^{-7} *M* (nuclei), suggesting that these were identical or similar enzymes. Peterson *et al.* (1965) found 11-HSD in the microsomal and nuclear fractions of rat brain. Sakai *et al.* (1992), however, found enzyme activity exclusively in brain and pituitary nuclei. Thus, the location of 11-HSD is not limited to the endoplasmic reticulum and may be distributed between subcellular organelles in a tissue- or cell-specific manner.

4. *Nucleotide Specificity*

The oxidoreductases that catalyze the transformations of steroid molecules are dependent on pyridine nucleotide coenzymes. These enzymes fall into three categories. The 3β-4-ene and 3α-4-ene hydroxysteroid dehydrogenases (Grosso and Unger, 1964) and 21-hydroxysteroid dehydrogenase (Monder and White, 1963, 1965) have dual nucleotide specificity, utilizing either NAD or NADP as cofactors; guinea pig liver and kidney 17β-hydroxysteroid dehydrogenases exist as particulate NAD-dependent and soluble NADP-dependent forms (Endahl *et al.*, 1960; Endahl and Kochakian, 1962); rat ovarian 20α-hydroxysteroid dehydrogenase (Wiest and Wilcox, 1961) and liver 5β-4-ene reductase (Tomkins and Isselbacher, 1954) are strictly NADP-dependent.

A survey of nucleotide specificity of 11-HSD in various tissues is presented in Table V. Rat liver enzyme has been reported to have dual nucleotide specificity, with NADP more effective than NAD (Hurlock and Talalay, 1959; Bush *et al.*, 1968; Koerner, 1966, 1969). In other tissues, including lung, kidney, placenta, intestinal mucosa, adipose tissue, striated muscle, and spleen, NADP was more effective than NAD, or NAD was not a cofactor. Two groups found that NAD and NADP were equally effective with enzyme from rat kidney (Mahesh and Ulrich, 1960) and human placenta (Meigs and Engel, 1961). Mercer and Krozowski (1992) have proposed that rat kidney contains distinct NADP- and NAD-dependent forms of 11-HSD. NAD was reported to be a better cofactor than NADP with 11β-dehydrogenase from rat mandibular gland. In one study with rat liver, NADPH was a better cofactor than NADH (Bush *et al.*, 1968). The data are consistent with a heterogeneous distribution of NAD- and NADP-responsive forms of 11-HSD.

5. *Kinetic Constants*

The K_m values summarized in Table VI are taken from a variety of sources and extend over a 1000-fold range in the oxidative direction, extending from 0.1 μ*M* for mouse spleen microsomes (Deckx and DeMoor, 1966) to 172 μ*M* for mouse liver microsomes (Burton, 1965). Direct comparison of the various values cannot be readily made, because of the large number of variables that must be taken into consideration: steroid substrate, pH, temperature of incubation, tissue preparation, tissue fraction, cofactor concentration. The broad variability persists even if only microsomes measured at 37°C and pH 7.4 are compared (Burton, 1965; Bernal *et al.*, 1980). The K_m values for cortisone reduction also extend over a wide range.

TABLE V
COENZYME SPECIFICITY OF 11-HSD

Tissue	F → E[a]	E → F[a]	Citation[b]
Rat liver	NADP (NAD not tried)	—	(1)
	NADP > NAD	NADPH > NADH	(2)
	NADP > NAD	—	(3–5)
Rat lung	NADP (NADH not tried)	NADPH (NAD not tried)	(6)
	NADP (NAD inactive)	—[c]	(4)
Rat kidney	NADP = NAD	—[c]	(7)
	NADP > NAD	—[c]	(4,8)
Human placenta	NADP = NAD		(9)
	NADP > NAD	—[c]	(10)
Mouse striated muscle	NADP (NAD inactive)	—[c]	(11)
Bovine striated muscle	NADP (NAD inactive)	—[c]	(11)
Human intestinal mucosa	NADP (NAD not tried)	—[c]	(12)
Human adipose	NADP > NAD	—[c]	(13)
Rat submandibular Gland	NAD > NADP	Not tried	(14,15)
Rat spleen	NADP > NAD	NADPH (NADH not tried)	(16)

[a] F, cortisol; E, cortisone.

[b] (1) Koerner (1969); (2) Bush *et al.* (1968); (3) Hurlock and Talalay (1959); (4) Koerner (1966); (5) Koerner and Hellman (1964); (6) Nicholas and Lugg (1982); (7) Mahesh and Ulrich (1960); (8) Kobayashi *et al.* (1987); (9) Meigs and Engel (1961); (10) Osinski (1960); (11) Sweat and Bryson (1960); (12) Burton and Anderson (1983); (13) Weidenfeld *et al.* (1982); (14) Hoyer and Moller (1977); (15) Furguson and MacPhee (1975); (16) Deckx and DeMoor (1966).

[c] NADPH had little or no effect in the reductive direction.

6. *pH Optimum*

The recorded values for the pH optimum of 11β-hydroxysteroid dehydrogenase, like the kinetic constants, vary broadly when measured in the oxidative or reductive directions. Oxidation of cortisol to cortisone by microsomes of mature rat liver was optimal at about pH 10 (Bush *et al.*, 1968; Koerner, 1969). Fetal mouse liver had a reported pH optimum of 8 (Michaud and Burton, 1977). The value for salivary gland homogenate was pH 7.6 (Furguson and MacPhee, 1975). Human placenta homogenate was reported by one laboratory to optimally oxidize cortisol in the pH range 8 to 9 (Osinski, 1960). Another laboratory reported a maximum above pH 10, with a plateau between pH 7 and 8

TABLE VI
MICHAELIS CONSTANTS REPORTED FOR 11-HSD IN VARIOUS TISSUES

Tissue	Fraction[a]	Variable substrate[b]	pH	K_m (μM)	Citation[c]
Rat liver	mc	Cortisol	7.4	16.4	(1)
Guinea pig liver	mc	Cortisol	7.4	27.1	(1)
Rat liver	mc	Cortisol	7.4	30	(2)
Rat liver	mc	Cortisol	8.6	17.6	(3)
Rat liver	mc	Corticosterone	8.5	9.2	(3)
Rat liver	mc	Corticosterone	8.6	0.22	(4)
Rat liver	nc	Corticosterone	8.6	0.27	(4)
Mouse liver	mc	Cortisol	7.4	172	(5)
Mouse fetal liver	mt	Cortisol	8.0	10	(6)
Rat lung	hom	Cortisol	7.4	4.1	(7)
Rat lung	mc	Cortisol	7.4	1.7	(7)
Mouse spleen	mc	Corticosterone	10	0.11	(8)
Mouse spleen	mc	Cortisol	10	0.23	(8)
Human adipose	hom	Cortisol	7.2	0.6	(9)
Human placenta	Mince	Cortisol	7.4	3.0	(10)
Human placenta	mc	Cortisol	7.4	0.3	(11)
Human decidua	mc	Cortisol	7.4	3.2	(11)

[a] mc, microsomal fraction; nc, nuclear fraction; mt, mitochondrial fraction; hom, homogenate.

[b] Constant cosubstrate was NADP.

[c] (1) Bush *et al.* (1968); (2) Koerner and Hellman (1964); (3) Monder and Lakshmi (1989a); (4) Murphy (1979b); (5) Burton (1966); (6) Michaud and Burton (1977); (7) Nicholas and Lugg (1982); (8) Deckx and DeMoor (1966); (9) Weidenfeld *et al.* (1982); (10) Kobayashi *et al.* (1987); (11) Bernal *et al.* (1980).

(Bernal *et al.*, 1980). Spleen microsomes were maximally effective at about pH 10 (Deckx and DeMoor, 1966). In the reverse direction, few values were available. The range was nevertheless broad, embracing values from pH 6.5 to 7.0 (Michaud and Burton, 1977; Deckx and DeMoor, 1966; Bush *et al.*, 1968).

The cause of such a wide range of values is not immediately apparent. That the method of preparation of the tissue may have played a role is suggested by data reported by Monder and Lakshmi (1989a). Freshly prepared rat liver microsomes generated a pH-activity profile with a maximum at pH 10 similar to what was reported by most investigators (Bush *et al.*, 1968; Koerner, 1969; Koerner and Hellman, 1964; Deckx and DeMoor, 1966). When briefly exposed to detergent, a profile resembling that obtained by Bernal *et al.* (1980) was obtained, with a plateau between pH 7 and 8, and a maximum at more alkaline values.

Varying conditions yielded distinctive pH-activity curves that were less a reflection of the intrinsic property of the enzyme than a composite reflection of the environment of the enzyme and its prior treatment.

D. Effects of Hormones

1. *Androgens and Estrogens*

In rats, there is a sex-dependent difference in 11-HSD of liver (Lax *et al.*, 1978, 1979) and kidney that favors males (Smith and Funder, 1991). Consistent with this observation is the strong evidence that 11-HSD is affected by the administration or withdrawal of sex steroids. The reduction of cortisone to cortisol by male rat lung is diminished after castration (Nicholas and Lugg, 1982). Gonadectomy may decrease liver 11-HSD in male rats as well, but appears to have no effect on female rats (Lax *et al.*, 1979). The introduction of testosterone to gonadectomized males is reported to bring the liver enzyme up to normal, whereas estradiol almost completely suppresses liver activity in male and female rats. Testosterone can increase the activity of female liver to about the level of normal male liver (Lax *et al.*, 1979). The response of different organs may not, however, be uniform. It has been reported that 11-HSD in genital skin fibroblasts of squirrel monkey is inhibited by testosterone (Hammami and Siiteri, 1990). These studies suggest that male and female steroids have opposite effects on 11-HSD expression.

The effects of estradiol and testosterone on liver 11-HSD of hypophysectomized rats are different from their effects on gonadectomized animals. Inhibition of activity by estradiol is suppressed, whereas testosterone raises the level of activity somewhat above normal. Hypophysectomy appears to release an endogenous suppression in females, raising the activity above that of comparable male rats. The effects of hypophysectomy are complicated, since this process eliminates numerous peptide and steroid hormones. In general, ablation of the pituitary results in loss of sex steroid dependence of liver enzymes of steroid metabolism (Gustafsson and Stenberg, 1976). The effects have been attributed to growth hormone. However, no studies have yet been performed on the growth hormone dependence of 11-HSD.

The response of neonatal rat testis to androgen and estrogen administration was similar to that seen in livers of hypophysectomized rats. Estradiol lowered 11-HSD activity and testosterone had no effect (Ghraf *et al.*, 1975b). It would therefore be expected that differences in

the level of 11-HSD would be seen in the two sexes in tissues that are responsive to sex steroids. The administration of the antiestrogen MER-25 to pregnant baboons prevented the increase in the capability of the placenta to oxidize cortisol to cortisone with advancing gestation, indicating that estrogen regulates the activity or synthesis of placental 11-HSD (Pepe and Albrecht, 1987). This observation was tested directly by increasing placental estrogen production and showing a similar increase in the extent of oxidation of cortisol to cortisone (Pepe *et al.*, 1988; Baggia *et al.*, 1990).

In the rat kidney, the effects of gonadectomy are unclear. In one study (Ghraf *et al.*, 1975b), it was found that female animals respond to ovariectomy by developing normal male 11-HSD levels, whereas male animals subjected to castration retained the activity unchanged. Hypophysectomy established normal male activity in both sexes. For female animals, therefore, hypophysectomy is equivalent to gonadectomy. In another study (Smith and Funder, 1991), the opposite was found. Gonadectomy decreased renal 11-HSD in males and had no effect on females.

Differences in 11-HSD activity in the two sexes are increased after puberty in normal rats. In liver and kidney, the female values are lower, because of the suppressive effect of estradiol. Values for the gonads, where concentrations of the sex steroids are predictably high, are consistent with those for other tissues (Hoff *et al.*, 1973).

In perfused male rat lung, castration decreased reduction of cortisone to cortisol (Nicholas and Lugg, 1982). Therefore the effects of sex steroids on 11-HSD are broad ranging and affect the enzyme in several organs. Adrenal 11-HSD measured in the oxidative direction is highest in the meadow vole during the winter, and is depressed during the breeding season, which is associated with an increase in corticosterone and increased adrenal size. It has been suggested that the circumannual effect is caused by seasonal ingestion of phytoestrogens (Unger *et al.*, 1978).

2. *Corticosteroids*

Glucocorticoids may intervene in their own metabolism by influencing the activity of 11-HSD. Some indirect suggestion that glucocorticoids affect lung 11-HSD is based on the observation that stress increases the activity of rat lung 11-HSD in the reductive direction (Nicholas and Lugg, 1982). Cortisol, which regulates the mitosis, maturation, and mortality of lymphocytes, also affects the level and direction of their 11-HSD activity. Thymic cells of mice pretreated with cortisol for 9 days showed increased activity in the oxidative direction

and no change in the reductive direction (Dougherty *et al.*, 1960). Placental 11-HSD of the baboon may be resistant to corticosteroid hormones. Serum cortisone did not alter the level of placental 11-oxoreductive activity and possibly decreased 11β-dehydrogenase (Pepe and Albrecht, 1985a). Although it is unlikely that the effect is due to direct inhibition by cortisone of enzyme activity, the addition of pregnenolone (250 n*M*), progesterone (25 n*M*) or cortisone (250 n*M*) to human or baboon placental homogenates inhibited oxidation of cortisol to cortisone (Pepe and Albrecht, 1984a). Bernal *et al.* (1982) found no changes in human placental 11-HSD taken after elective cesarean section, or after spontaneous and induced labor. However, injections of dexamethasone into pregnant rhesus monkeys increased cortisol-to-cortisone conversion by the placenta (Althaus *et al.*, 1982).

Isolated perfused fetal rabbit lung oxidized cortisol, but the reverse reaction was minimal (Torday *et al.*, 1976). Lugg and Nicholas (1978) found that the development of 11-HSD in the lung of the fetal rabbit *in vivo* is not affected by dexamethasone after direct injection into the fetus. Fetal rat lung, however, responded to betamethasone, another glucocorticoid analog, with an increase in 11-HSD reduction. *In vitro*, cortisol stimulated growth of fetal human lung cells. This corresponded with increased net conversion of cortisone to cortisol (Smith *et al.*, 1973). Abramovitz *et al.* (1982) showed that fetal lung cells diverged during growth in tissue culture into populations of epithelial cells and fibroblast-like cells which could be cultured separately. The former preferentially oxidized cortisol to cortisone; the latter, which was the dominant surviving cell type, reduced cortisone to cortisol. These findings may explain an apparent contradiction between findings of Smith *et al.* (1973) described above and those of Pasqualini *et al.* (1970a) and Murphy (1978), who found that fetal lung primarily catalyzed cortisol oxidation, as did all other fetal tissues. Reduction of cortisone increased with lung maturity. This developmental pattern also applies for the fetal rat (Smith, 1978). The reduction of cortisone by fetal rabbit lung was reported by Giannopoulos (1974). Mature rat lung rapidly reduced cortisone to cortisol (Nicholas and Kim, 1975). The suggestion that the direction of 11-oxygen metabolism in lung is determined under physiological conditions by the nature of the cell population remains to be tested.

3. *Thyroid*

There have been several studies published on the effects of thyroid hormone on 11-HSD. Species specificity has been observed on the effects of thyroxine on the oxidation of cortisol to cortisone by liver.

Thyroxine administration causes a decrease in male rat liver (Koerner and Hellman, 1964; Lax *et al.*, 1979) that is only apparent after 7 days of continuous exposure. In contrast, hyperthyroid humans respond with increased hepatic activity (Zumoff *et al.*, 1983; Hellman *et al.*, 1961; Gordon and Southren, 1977). Thyroidectomy or hypothyroidism reverses the response, resulting in increased activity in the rat (Koerner and Hellman, 1964) and decreased activity in humans (Zumoff *et al.*, 1983; Hellman *et al.*, 1961; Gordon and Southren, 1977). The effects of thyroid hormones are tissue specific. No changes occur in the kidney (Koerner and Hellman, 1964; Lax *et al.*, 1979; Smith and Funder, 1991) or in the reticuloendothelial system (Dougherty *et al.*, 1960). The proposal that thyroid hormones act by controlling the availability of pyridine nucleotides (Dougherty *et al.*, 1960) appears not to be borne out by experiment (Koerner and Hellman, 1964). Enzyme inhibitors are not formed (Koerner and Hellman, 1964). Whether thyroid hormones directly affect the level of enzyme is not known. It has been suggested that thyroid hormone controls the level of available testosterone, and thus indirectly influences 11-HSD.

4. *Other Hormones*

The activity of placental 11-HSD, which shows activity almost entirely in the oxidative direction, is not affected by prolactin, hCG, or ACTH *in vitro*. The cortisol and cortisone content of amniotic fluid of diabetic and nondiabetic women are identical. Therefore insulin, glucagon, and the various diabetogenic factors do not influence 11-HSD (Baird and Bush, 1960).

III. Developmental Biology and 11-HSD

A. Fetal Development

1. *Placental 11-HSD*

The level of active corticosteroid to which the fetus is exposed is crucial to its development and maturation. Too high exposure can lead to developmental disturbances. The placenta catalyzes the oxidation of the 11β-hydroxy groups of corticosteroids, both natural (Burton and Jeyes, 1968; Bernal and Craft, 1981; Giannopoulos *et al.*, 1982; Pasqualini *et al.*, 1970a; Waddell *et al.*, 1988) and synthetic (Levitz *et al.*, 1978), and thus provides a barrier to the transfer of active glucocorticoid to the fetus by converting the steroids to the biologically inactive

11-oxo form. In keeping with this role, reduction of 11-oxosteroids by placental 11-HSD is low or not detectable (Osinski, 1960; Bernal *et al.*, 1980; Murphy *et al.*, 1974; Murphy, 1979b; Kittinger, 1974). As a consequence of this overwhelming oxidative activity, relatively high proportions of 11-oxocorticosteroid metabolites appear in normal amniotic fluid and fetal cord serum (Osinski, 1960; Baird and Bush, 1960; Bro-Rasmussen *et al.*, 1962). When the placental barrier is bypassed, intra-amniotically administered cortisol is absorbed by the human fetus and is oxidized in individual organs slowly, resulting in long-term retention of active steroid (Murphy and Vedady, 1982). The proportion of cortisol relative to cortisone is greater in the amniotic fluid than the cord fluid and rises with gestation in humans (Murphy, 1977a). Chorionic membrane catalyzes a reduction of cortisone to cortisol (Murphy, 1977b; Bernal *et al.*, 1980) and may contribute to the rise in active steroid. Although the direction of metabolism of the 11-oxygen by placenta and chorion does not change during gestation, it is not known whether the levels of 11-HSD activity in these organs change. The few studies that have been performed with human placenta have yielded conflicting results. Giannopoulos *et al.* (1982) have reported quantitative changes in the capacity of placenta and decidua to interconvert cortisol and cortisone; Bernal *et al.* (1982) find no changes in placental corticosteroid metabolism during the terminal stages of pregnancy in the human, but find changes over the longer term. These results may not in fact be contradictory since the span of gestation studied in the two investigations were quite different, the former extending through the major part of pregnancy, and the latter considering only the terminal stages. Tanswell *et al.* (1977) have suggested that reductive capacity of the chorion is valuable for the fetus, because it represents a mechanism for regenerating cortisol for the fetus, possibly acting as an accessory adrenal gland.

2. *The Feto-Placental Unit*

The behavior of the placenta *in vitro* confirms that a highly effective barrier exists against the transfer of 11β-hydroxysteroids from mother to fetus. The ability of the human and primate feto-placental unit to efficiently oxidize cortisol to cortisone results in the transfer of little or no cortisol into the fetus (Althaus *et al.*, 1982), who is thus protected against the teratogenic actions of cortisol (Murphy *et al.*, 1974; Munck and Leung, 1977; Slikker *et al.*, 1982). The existence of this barrier also permits the fetus to retain autonomy over its own cortisol production (Murphy and Branchaud, 1983; Beitins *et al.*, 1972; Mitchell *et al.*, 1981, 1982). The timing of the increase in active corticosteroid level in

the maturing fetus is essential for creating an internal environment compatible with independent life (Murphy, 1977a). Synthetic steroids that are poor substrates for 11-HSD, such as dexamethasone, are transferred to the fetus largely unoxidized (Althaus *et al.*, 1982; Funkenhouser *et al.*, 1978; Anderson *et al.*, 1979). This process may have important pharmacological implications. Pepe and co-workers have studied the transplacental regulation of cortisol metabolism in pregnant old world monkeys. They have suggested that transuteroplacental corticosteroid metabolism may play an important role in the maturation of the pituitary–adrenocortical axis (Pepe and Albrecht, 1985b). Although transfer of cortisone (from maternal cortisol) to the fetus is extensive, little cortisone is converted to the active hormone (Mitchell *et al.*, 1982). Most of the cortisol available to the late gestation fetus, as illustrated with baboon and rhesus, is endogenous (Mitchell *et al.*, 1981; Althaus *et al.*, 1982; Pepe and Albrecht, 1984b), resulting from secretion of the maturing fetal adrenal.

3. *Fetal 11-HSD*

Fetal tissues contribute to the net oxidation of corticosteroids in the developing organism. Brain, gut, liver, and lung in the fetal mouse are all strongly oxidizing at 14 days of gestation. By 19 days, with birth approaching, the tissues show increasing capacity for reduction (Tye and Burton, 1980), in some cases evolving from net oxidation to net reduction. The capacity of the mouse liver to catalyze net reduction continues to increase after birth (Burton and Jeyes, 1968).

Other organs change their relative preference of direction of 11-oxidoreduction during development. The nonpregnant human uterus preferentially oxidizes cortisol to cortisone, but catalyzes the reverse process during early pregnancy (Murphy, 1977b). The net effect of the metabolic events catalyzed by 11-HSD in the fetus is the oxidation of cortisol to cortisone or corticosterone to 11-dehydrocorticosterone (Murphy, 1981; Pasqualini *et al.*, 1970a,b). The proportion of oxidized to reduced form decreases during gestation as 11-HSD in the liver (Michaud and Burton, 1977; Smith *et al.*, 1982) and lung (Nicholas and Lugg, 1982; Smith *et al.*, 1982; Smith, 1978) plays an increasingly important role in reducing the 11-oxo group of the steroid.

The changes in steroid oxidoreduction in the individual fetal organs are intimately connected with the maturational events that prepare the organism for birth and permit its subsequent independent existence (Liggins, 1976). Fetal lung has been the subject of intense study. Pulmonary differentiation is dependent on and accelerated by 11β-hydroxylated corticoids. Glucocorticoids induce synthesis and release

of surfactant and the differentiation of alveolar cells (Avery, 1975). Steroid effects on lung maturation have important clinical applications in the prevention or reversal of hyaline membrane disease (Liggins and Howie, 1972). Since the dominant metabolic transformation of glucocorticoids in the fetus is 11-oxidation, the ability of the lung to catalyze 11-oxoreduction is of particular significance. Conversion of cortisone or 11-dehydrocorticosterone to their respective 11-reduced forms is essential for lung differentiation (Torday, 1980; Giannopoulos, 1974; Murphy, 1981; Torday *et al.*, 1975; Drafta *et al.*, 1975) based on the criteria of lung size (Drafta *et al.*, 1975), cell growth (Torday, 1980; Smith *et al.*, 1973), phosphatidylcholine production (Drafta *et al.*, 1975; Torday *et al.*, 1975), and glycogen content (Smith *et al.*, 1982). The ability of fetal lung to reduce 11-dehydrocorticosteroids increases during gestation in rabbit (Dougherty *et al.*, 1960; Torday *et al.*, 1976; but see Hummelink and Ballard, 1986), human (Smith *et al.*, 1973), mouse (Burton and Turnell, 1968), and rat (Smith *et al.*, 1982). It is possible that in human lung the increase in reductive ability during gestation may in part be due to a large decrease in the 11β-dehydrogenase as well as an absolute increase in 11-oxoreductase. It has also been suggested that the driving force in the increase in 11-oxoreduction is an increase in NADPH (Torday *et al.*, 1976).

During the second trimester and early third trimester of pregnancy, 11-oxo steroids exceed 11-hydroxysteroids in the fetal circulation (Waddell *et al.*, 1988; Murphy and Diez d'Aux, 1972; Burton and Jeyes, 1968; Sowell *et al.*, 1971). Murphy *et al.* have documented the extensive catabolism of cortisol to cortisone in the human midterm fetus (Murphy, 1979b, 1981; Murphy and Branchaud, 1983). The magnitude of oxidation of cortisol and corticosterone is dependent on the combined metabolic actions of the placenta, its associated membranes, and the fetal tissues. The relative oxidative and reductive activities in many tissues change with time. In most tissues, irrespective of species, the oxidation of steroids dominates at midgestation. In late gestation, reductase activity is expressed in some tissues. It is not yet known whether the shifts in dehydrogenase–oxoreductase capabilities of some tissues are species specific, nor is it known for most organs when, during development, the expression of 11-HSD activity first appears.

Perinatal reduction is dominated by the lung and liver. In mouse liver net 11-reduction continues to increase after birth. The relative capabilities of each tissue to catalyze 11-oxidation or 11-reduction correlate well with the proportion of 11-oxo- to 11-hydroxysteroids in these tissues (Smith, 1978; Smith *et al.*, 1982). The ratio of reduced to oxidized steroid in tissues at critical stages of development may pro-

vide important clues to determining key molecular events necessary to complete development. There is no doubt that the presence of the physiologically appropriate level of active corticosteroid at the correct stage of development of fetal organs is essential for their proper development and function. In view of this and the known teratogenic effects of corticosteroids when administered in excess to experimental animals, there is ample reason to suspect that 11-HSD performs a crucial mediating role in fetal development.

B. Postnatal Development

1. *In Vivo Metabolism of Corticosteroids*

After birth, overall corticosteroid metabolism at C-11 is reductive. In some organs, such as uterus, parotid gland, colon, and kidney, metabolism continues to be predominantly oxidative. One consequence of the concurrent selective exposure of steroids to oxidative or reductive conditions in the various organs is the excretion of a mix of 11-oxo- and 11β-hydroxysteroid metabolites. In humans and primates, who excrete corticosteroid metabolites mainly by way of the kidney, measurement of urinary steroid metabolites provides an accurate reflection of the oxidoreductive balance. In other organisms, such as rats and mice, that utilize the gastrointestinal tract as the dominant excretory pathway for steroids, establishing the net balance of oxidation and reduction is far more difficult, and has not yet been successfully accomplished.

One approach to the study of murine steroid metabolism utilizes biliary steroids. In rats, about 90% of corticosterone metabolites are recovered from bile (Gustafsson and Gustafsson, 1974), reflecting primarily hepatic metabolism (Eriksson and Gustafsson, 1971). Most identified metabolites contain the 11β-hydroxy group, suggesting that liver metabolism at C-11 is primarily reductive *in vivo*.

In humans, the metabolites of endogenously produced cortisol are excreted into the urine as a mixture of products at different levels of reduction and oxidation (Peterson *et al.*, 1955). These include metabolites reduced in ring A (tetrahydrocortisol, tetrahydrocortisone), and ring A-reduced metabolites further reduced at C-20 (cortols, cortolones); of those metabolites in which oxidation dominates, the major examples are the cortoic acids, C_{21} steroids containing a carboxylic acid group at C-21 (Monder and Bradlow, 1980). There are additionally significant amounts of metabolites resulting from the loss of the ketol side chain, and a number of minor metabolites.

2. *Corticosteroid Metabolites in Health and Disease*

Cortisol in normal humans, male and female, has a biological half-life of 95 to 130 min (Peterson *et al.*, 1955). Cortisone has an average plasma biological half-life of 28 min. This is due in part to its rapid conversion to cortisol, and in part to the greater susceptibility of cortisone to catabolism. Its reduction to cortisol, mediated by 11-HSD, is the basis for its pharmacological action; the leaking away of cortisone by competing catabolic reactions is the reason that cortisone is a less potent pharmacological agent than cortisol. That cortisol is oxidized at C-11, i.e., that the oxidation–reduction process is physiologically freely reversible, is revealed by the profile or urinary metabolites. The ratio of the major metabolic products tetrahydrocortisol (THF), allotetrahydrocortisol (5α-THF, ATHF) and tetrahydrocortisone (THE), expressed as (THF + ATHF)/(THE) = R, has been used as a measure of the physiological oxidoreductase activity at C-11. Numerous studies have confirmed that alterations in physiological status cause significant changes in this ratio. Table VII presents a qualitative assessment of the effects of a variety of conditions on the value of R relative to that of normal subjects, whose values range from 0.5 to 2. Irrespective of the nature of the illness, the proportion of 11-reduced metabolites increased relative to control (presumably normal) populations, with few exceptions. The changes were not large, rarely exceeding 50%, and are insufficiently great in magnitude to distinguish whether changes in the level of enzyme or pyridine nucleotide are rate limiting, or whether the difference can be attributed to a selective redistribution of 11-oxosteroids between tetrahydro and pentahydro metabolites (Bradlow *et al.*, 1968; Zumoff *et al.*, 1968b). Zumoff *et al.* have shown that while (THF + ATHF)/(THE) increased in cirrhosis (Zumoff *et al.*, 1967) the total value of C-11 hydroxy/C-11-oxo, including all urinary steroids, was unchanged from normal (Zumoff *et al.*, 1968a), suggesting increased conversion of THE to cortolones. Where the secretion of cortisol from the adrenal results in peripheral accumulation great enough to exceed the ability of the organism to dehydrogenate at C-11, the urinary cortisol/cortisone and THF/THE ratios increase (Baulieu and Jayle, 1957).

During postnatal development, the R values change from ca.0.1 at birth to approximately unity, as Fig. 3 shows. The early low values of this ratio are the consequence of the fact that in the recently born infant, 11-dehydrogenation is highly active, resulting in the excretion of THE, but little THF. This pattern also occurs in primates other than human (Pepe and Townsley, 1976). The proportion of THE and THF

TABLE VII
EFFECT OF DISEASE ON THE PROPORTION OF URINARY 11β-HYDROXY TO 11-OXO METABOLITES

Condition	Effect[a]	Citation[b]
Cushing's disease or ACTH	$R_C > R_N$	(1-5)
Infection, nonspecific illness	$R_C > R_N$	(6,7)
Rheumatic disorders	$R_C > R_N$	(7,8)
Cirrhosis	$R_C > R_N$	(9)
Essential hypertension	$R_C > R_N$	(10,11)
Chronic myelogenous leukemia	$R_C = R_N$	(12)
Adrenal carcinoma	$R_C > R_N$	(13)
Schizophrenia	$R_C = R_N$	(14)
Hypothyroid	$R_C > R_N$	(15)
Hyperthyroid	$R_C < R_N$	(15)
Endogenous depression	$R_C < R_N$	(16)
Chronic renal failure	$R_C > R_N$	(17)
Anorexia nervosa	$R_C < R_N$	(18,19)

[a] R = (THF + ATHF)/THE [tetrahydrocortisol + allotetrahydrocortisol)/tetrahydrocortisone]. R_C = subjects with designated condition. R_N = normal or control subjects.

[b] (1) Gray *et al.* (1962); (2) Bailey and West (1969); (3) Peterson and Pierce (1960); (4) Bush and Willoughby (1957); (5) Kornel (1970); (6) Zumoff *et al.* (1974); (7) Ichikawa (1966); (8) Pal (1967); (9) Zumoff *et al.* (1967); (10) Kornel *et al.* (1969); (11) Walker *et al.* (1991); (12) Gallagher *et al.* (1965); (13) Fukushima *et al.* (1960); (14) Romanoff *et al.* (1957); (15) Hellman *et al.* (1961); (16) Murphy (1991); (17) Walker and Edwards (1991); (18) Vierhapper *et al.* (1990); (19) Vanluchene *et al.* (1979).

shifts to the dominant postnatal ratio of 1–2 during the first year of life (Daniilescu-Goldinberg and Giroud, 1974; Savage *et al.*, 1975; Blunck, 1968; Kraan *et al.*, 1980); C. H. L. Shackleton, personal communication). The relationships between F and E in serum and amniotic fluid during the last trimester of pregnancy are similar to those of THF and THE (Noma *et al.*, 1991). So strong is the oxidation pressure in infants, that the blood F/E ratio will remain <1 even after intravenous administration of high concentrations of cortisol (maternal F/E = 11) (Buus *et al.*, 1966). The change in the 11-hydroxysteroid/11-oxosteroid ratio during early development is in accord with the changes in the increasing ability of 11-HSD to catalyze 11-reduction relative to 11-oxidation.

No data are available for the prenatal metabolism of corticosterone in humans. At the earliest known age examined, 1 year, the value for (THB + ATHB)/THA indicated a strong preference for the reduced

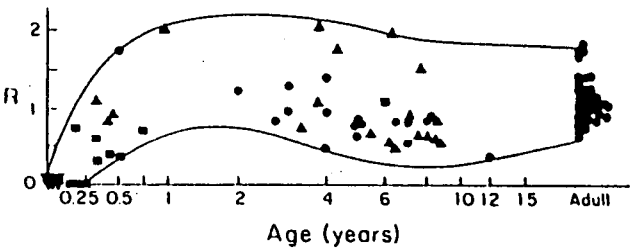

FIG. 3 The effect of age on the value of R = (THF + 5α THF)/THE. Modified from Monder and Shackleton (1984).

forms, THB and alloTHB (Peterson and Pierce, 1960; Savage *et al.*, 1975; Kornel *et al.*, 1969; Blunck, 1968). At every age, R_B exceeded R_F by two- to eightfold. The values for R_B fluctuated over a wide range between laboratories, and so from the limited data available, it is not possible to draw conclusions about age-related trends.

3. *C-11 Metabolism in Specific Organs*

Continuing the prenatal trend, 11-HSD increases in several organs during early postnatal development in the mouse (de Moor and Deckx, 1966) and rat (Hoff *et al.*, 1973), then decreases. In the mouse, 11-HSD, measured as oxidation of corticosterone at pH 10.5, rises from birth to 10 weeks of age in spleen, kidney, and liver, suggesting that it is due to some coordinated process, then declines to intermediate values. Development in the rat liver is qualitatively similar. Maximum value of cortisol oxidation occurs at 30 days of age, followed by a decline. Thus, in all species, alterations in the interconversion of corticosteroids at C-11 initiated in the fetus continue after birth, each organ following a unique pattern (Mitchell *et al.*, 1981; Pepe, 1979; Krozowski *et al.*, 1990; Moisin *et al.*, 1992).

IV. ARE 11-DEHYDROCORTICOSTEROIDS BIOLOGICALLY ACTIVE?

Cortisone is converted faster than cortisol to inactive metabolites: It binds poorly to glucocorticoid receptors (G. G. Rousseau *et al.*, 1972) under optimal experimental conditions and probably not at all under physiological conditions. On the basis of these observations it would not be predicted that 11-dehydrocorticosteroids have significant bio-

logical activity. There are, however, several observations that suggest otherwise. Barseghian *et al.* (1982) reported that cortisone, but not cortisol, strongly inhibited glucose- and arginine-induced insulin secretion. Berliner and Ruhmann (1966) found that though cortisol inhibited fibroblast growth in tissue culture, cortisone increased growth. In guinea pig isolated ileum, cortisone antagonized $GABA_A$ receptor-mediated contractile responses to applied GABA, in contrast with the enhancing effect of cortisol (Ong *et al.*, 1990). Souness and Morris (1990) have found that 11-dehydrocorticosterone caused significant kaluresis in male adrenalectomized rats and blocked the sodium ion retention caused by aldosterone. Souness and Morris (1991) have also found that, though 11-dehydrocorticosterone was not reduced to corticosterone by toad bladder preparations, the steroid nevertheless blocked aldosterone-stimulated short-circuit current.

There is one report, as yet unconfirmed, that 11-dehydrocorticosteroids have intracellular activity, mediating salt metabolism in the nasal gland of the domestic duck (Sandor *et al.*, 1983). The molecular basis for this observation is obscure. Membrane-associated events may explain the effects of cortisone on ileum contractile responses (Ong *et al.*, 1990), and possibly on other cortisone-mediated processes. The number of reports of 11-dehydrocorticosteroid activity are few, and none have been independently confirmed. Nevertheless, the examples cited support the possibility that oxidation of corticosteroid at C-11 may not be exclusively inactivating, and may generate physiologically significant metabolites.

V. 11-HSD in Lower Vertebrates

The presence of 11β-hydroxy- and 11-oxosteroids in animals as diverse as fish (Chan and Yeung, 1989; Gottfried, 1964), birds (Holmes *et al.*, 1974), and the platypus (McDonald *et al.*, 1988) indicates that 11-HSD serves an important function in nonmammalian vertebrates. Both cortisol and cortisone are found in salmon blood (Idler *et al.*, 1959a,b) and though oxidation may occur, cortisone does not appear to be effectively reduced to cortisol (Idler and Truscott, 1963). These conversions are probably extrahepatic, possibly occurring in the anterior kidney (Columbo and Bern, 1970), since in a wide range of bony fish there was no evidence of liver 11-HSD (Columbo *et al.*, 1972; Monder and Lakshmi, 1989a). However, generalizations about the role a particular organ in fish may play in 11-oxoreduction must be qualified. For example, in the ganoid fish, *Amia calva*, the anterior kidney was deficient in 11-HSD, unlike that of another ganoid, *Lepisosteus osseus* (Columbo *et al.*, 1972). The appearance in bile of cortisone and tetrahydrocortisone after injection of cortisol into trout (Truscott, 1979) or salmon (Donaldson and Fagerlund, 1972) is consistent with an active hepatic 11-HSD (Kime, 1978) in some species of teleost. The resolution of the source of 11-oxidoreduction in fish is of additional importance because C-11 steroids may be the source of the teleost androgens 11β-hydroxy- and 11-oxotestosterone (Idler and MacNab, 1967; Leitz and Reinboth, 1987; Rosenblum *et al.*, 1985). The somewhat more advanced African lung fish *Protopterus*, in contrast, is incapable of oxidizing the C-11 hydroxy group (Idler *et al.*, 1972). Early work on the occurrence of 11-oxygenated steroids in lower vertebrates have been summarized by Gottfried (1964).

Direct measurement of 11-HSD in livers of vertebrates has been made by Monder and Lakshmi (1989a). No 11β-dehydrogenase was detected in the liver microsomes of the frog, toad, mud puppy, shark, and several birds. In contrast, all mammals had activity. In the reductive direction, activity was present only in the livers of dogfish, birds, and mammals. Amphibians and teleosts had no detectable enzyme.

The duck nasal gland provides an interesting example of a system in which 11-oxidation may activate a steroid. Marina birds have a specialized organ, the nasal salt-gland, which protects them against the high salinity of ingested sea water. These glands concentrate and excrete the excess salt by a mechanism that is corticosteroid dependent. The endogenous corticosteroid, corticosterone, is rapidly oxidized to 11-dehydrocorticosterone by the nasal gland *in vivo*, *in vitro*, or by cell-free homogenates of the gland (Takemoto *et al.*, 1975; Sandor *et al.*, 1977). The glucocorticoid receptor, or an enzyme closely associated with the receptor, converts the specifically bound corticosterone to 11-dehydrocorticosterone (Sandor *et al.*, 1977, 1983; Sandor and Mehdi, 1980), which is transported to the nucleus. It is proposed that the receptor binds corticosterone, and its activation requires oxidation of the steroid at C-11.

VI. The Forms of 11-HSD Expression: Uniqueness or Multiplicity?

A. On the Question of Reversibility

The evidence presented thus far provides us with a picture of a functionally highly flexible enzyme, capable of adapting to net oxidation or net reduction depending on changing circumstances of age, health, state of gestation, and hormonal status. This remarkable

adaptive process engages the whole animal, and every organ within in unique and distinctive ways. The range of these processes is determined to some degree by genetic endowment (Nguyen-Trong-Thuan *et al.*, 1971). However, within these proscribed limits, the ability of the enzyme to respond to changing conditions is so striking that a closer look at it is justified. How is it possible for a single, presumably well-characterized enzyme to express itself as a net dehydrogenase under some conditions, and as a reductase under others?

A number of mechanisms have been proposed to account for the differential behavior of 11-HSD in various tissues and the changes in level of activity and in the directional characteristics of oxidoreduction that occur during development. Several rely on the properties of 11-HSD as a reversible pyridine nucleotide dependent oxidoreductase. Nicholas and Lugg (1982) and Torday *et al.* (1976) have postulated that the changing NADP/NADPH ratio is the driving force that determines the relative proportion of 11β-hydroxy- to 11-oxosteroid in lung in response to stress, castration, or adrenalectomy. Dougherty *et al.* (1960) utilized a similar mechanism to explain the appearance in immature lymphocytes of increased net 11-oxoreduction of cortisol subsequent to the introduction of triiodothyronine.

Other investigators have presented evidence that tissue specific changes in 11-HSD activities are not determined by the oxidation–reduction state of the tissue (Bernal *et al.*, 1980; Bernal and Turnbull, 1985) since they occur when nucleotide cofactors are not rate limiting. The effects of thyroxine in the rat persisted when pyridine nucleotide cofactors were not limiting (Zumoff *et al.*, 1983; Hellman *et al.*, 1961; Koerner and Hellman, 1964).

Product inhibition has been shown not to be responsible for the divergent effects of 11-HSD. Cortisol, even at 100-fold excess did not inhibit 11-oxoreductase activity (Bernal *et al.*, 1980). In the oxidative direction, neither NADPH nor 11-dehydrocorticosterone inhibited rat liver 11-HSD (Monder, 1991a).

An alternative hypothesis based on environmental perturbations is that the equilibrium ratio depends on pH. Changes in pH can theoretically affect the corticosteroid–11-dehydrocorticosteroid ratio, since the equilibrium of the overall redox reaction is dependent on the concentration of protons. The implementation of this hypothesis depends on knowledge of the equilibrium constant of the reaction and the local pH in the environment of the enzyme. Both are unknown. Changes of pH in the physiological range are not large enough to affect the 11-hydroxy/11-oxo ratio to a major degree (Lakshmi and Monder, 1985b; Monder and Shackleton, 1984). Any large local pH change that would persist for a sufficiently long time to alter the direction or magnitude of 11-HSD catalyzed reaction would adversely affect other processes in the endoplasmic reticulum.

Of the "environmental" hypotheses, the most likely mechanism accounting for small changes in oxidation–reduction properties is the one that proposes changes in the ratio of pyridine nucleotide cofactors. This may be an occasional mechanism for rapid, local perturbations in 11-oxoreduction. However, it can be readily shown that the ratios of reduced to oxidized pyridine nucleotides [NADPH/NADP] would have to be unrealistically high or low in order to account for the apparent extreme values of 11β-hydroxy/11-oxo in many tissues during development. Under physiological conditions, where the changes in 11-hydroxy/11-oxo in the whole organism based on urinary excretion patterns are not large, the relative proportion of reduced-to-oxidized cofactors may contribute to the behavior of the steroid at C-11. However, so many reactions occur in which both steroids and other biological substances require pyridine nucleotide cofactors within a cell, that is is not often that a circumstance arises where the machinery of the cell becomes entirely subservient to the metabolic requirements of a single molecule for any finite interval of time. Therefore, it is metabolically more likely and less disruptive for the cell to have developed other more specific mechanisms to control the interconversion of corticosteroids at C-11.

The possibility that the diverse behavior of 11-HSD in tissues is due to distinct, though related, enzymes has been considered by a number of investigators. In general, the view expressed has been that variants of 11-HSD are present in different tissues, representing forms with distinct kinetic properties that express behavior favoring reduction or oxidation. A model for this kind of system is glyceraldehyde phosphate dehydrogenase, in which different isozymes dominate in various tissues, and which have structural characteristics that lead to its preferential reduction to triose phosphate or oxidation to diphosphoglyceric acid (Kaplan, 1968). Thus the placental and decidual 11-HSD may be isozymes (Bernal *et al.*, 1980) as may also be true of the lung (Nicholas and Lugg, 1982) and liver (Bush and Mahesh, 1959b) enzymes.

B. Characteristics of Microsomal 11-HSD

1. *Latency*

The catalytic activity of the 11β-dehydrogenase component of 11-HSD is not fully expressed in liver microsomal preparations. Treatments that disrupt or alter the structure of the microsomal matrix,

such as phospholipase, detergent, and elevated pH, release latent enzyme activity. These processes, by altering membrane integrity, probably make the active site of 11β-dehydrogenase more accessible to its substrate (Gunderson and Nordlie, 1975). Latency of 11β-dehydrogenase, first observed in rat liver microsomes (Lakshmi and Monder, 1985b), occurs in the livers of other species, as well (Monder and Lakshmi, 1989a). This phenomenon is not unique. Other membrane-based enzymes express latency (Gunderson and Nordlie, 1975; Stetten and Burnett, 1967; Ernster and Jones, 1962; Schulze and Speth, 1980). It is possible that this property is a physiologically significant mechanism for controlling the expression of enzyme activity. In rat liver microsomal preparations, 11-oxoreductase activity is initially fully expressed without the intervention of latency releasing conditions (Lakshmi and Monder, 1985b). The latency behavior of hepatic 11β-dehydrogenase and 11-oxoreductase are therefore different.

2. *Energy of Activation*

The temperature dependence of enzyme activity can reveal much about the environment of the enzyme. The relationship of temperature and enzyme activity has been shown to adhere to thermodynamic principles and reflect the environment of the enzyme. The energy of activation is discrete in a homogeneous environment. If, however, the environment shows discontinuities, the energy of activation of an enzymatic process will show corresponding discontinuities should the activity be dependent on the structure of that environment (Raison *et al.*, 1971; Kumamoto *et al.*, 1971.

The energy of activation (E_a) of microsome-bound 11-dehydrogenase is continuous over the entire physiological temperature range and has the same value as the soluble enzyme. In contrast, microsomal 11-oxoreductase shows a discontinuity in E_a at 23°C, which is no longer present when the enzyme is solubilized, or when the microsomal lipid matrix is disrupted with phospholipases. The discontinuity coincides with a phase change in the matrix structure.

The differences between reductase and dehydrogenase with respect to latency and activation energy indicate that both activities are in distinct environments within the microsomal membrane. When they are solubilized, these environmental differences are removed, and the behavior of the enzymes reflect this (Lakshmi and Monder, 1985b).

3. *Enzyme Stability*

The relative stabilities of 11β-dehydrogenase and 11-oxoreductase provides an additional distinguishing criterion. Oxidation is the more

stable activity. With freshly prepared rat liver microsomal preparations, oxidation proceeds undiminished for at least 2 h at 37°C, whereas reductase is inactivated within 10 min.

We conclude from the literature and our own observations that there is sufficiently wide diversity in the properties and behavior of 11-HSD derived from different sources to be suggestive of multiple enzyme forms. The physicochemical and kinetic characteristics of the enzyme are also consistent with independent 11β-dehydrogenase and 11-oxoreductase sites. To address this question of enzyme multiplicity, it is necessary to review the molecular properties of 11-HSD. First, however, we will examine how the clinical evidence contributes to our understanding of its properties and physiological functions.

VII. Clinical Studies

A. 11β-Dehydrogenase Deficiency

1. *Apparent Mineralocorticoid Excess*

In humans, an "experiment of nature" has provided insight into the probable function of 11-HSD in at least one organ, the kidney. A disease apparently unique to children was described in the 1970s with a clinical picture consisting of low renin activity, low aldosterone production, hypokalemia, and severe hypertension (Ulick *et al.*, 1977; Werder *et al.*, 1975; Ramirez *et al.*, 1979; Winter and McKenzie, 1977). The first completely described patient with this condition was a Zuni Indian girl in whom the diagnosis was made at the age of 3 years (New and Levine, 1977; New *et al.*, 1977). Urinary cortisol and deoxycorticosterone metabolites were below normal and were not increased after ACTH stimulation. Glucocorticoid administration exacerbated the hypertension, suggesting that it was in some manner linked to endogenous cortisol.

Extreme sodium deprivation lowered blood pressure, possibly by stimulating the rate of conversion of corticosterone to aldosterone (Haning *et al.*, 1970). High doses of spironolactone, a potassium-sparing diuretic that acts via blockade of the mineralocorticoid receptor, also produced normalization of blood pressure, and on this regimen, plasma renin activity rose. Substitution of triamterene, a potassium-sparing diuretic that does not affect the mineralocorticoid receptor, failed to ameliorate blood pressure.

In vivo measurement of transcolonic electrical potential difference

in the patient was consistent with mineralocorticoid effect seen in patients with primary hyperaldosteronism; the potential difference was increased with hydrocortisone administration, and diminished with spironolactone administration (New *et al.*, 1982). Sensitivity of glucocorticoid receptors was normal as assayed in lymphocytes (Bigger *et al.*, 1972). Bioassays performed to demonstrate the presence of a steroid hormone in the patient's serum capable of causing sodium retention revealed no mineralocorticoid effect (Marver and Edelman, 1978; Blair-West *et al.*, 1962; Sennett *et al.*, 1975; Adam *et al.*, 1978; Baxter *et al.*, 1976). Because the symptoms and response to treatment were consistent with aldosteronism, despite the low circulating levels of the steroid, the syndrome was referred to as "apparent mineralocorticoid excess" (AME). This designation appears to have gained general acceptance.

Patients with AME have shown (*a*) low rate of cortisol turnover, with approximately twice the disappearance time of radiolabeled tracer steroid compared with that of a normal subject; (*b*) low peripheral plasma ACTH levels; (*c*) normal CBG concentration; (*d*) greatly diminished level of urinary metabolites of cortisone compared with those of cortisol; (*e*) no production of tritiated water after infusion of 11α-[^{3}H]cortisol, suggesting a defect in the oxidative component of the 11β-hydroxysteroid dehydrogenase (Ulick *et al.*, 1979); (*f*) normal metabolism of cortisone to cortisol, suggesting an intact reductive component of 11β-hydroxysteroid dehydrogenase (Ulick *et al.*, 1979; Monder *et al.*, 1986); and (*g*) an abnormal increase in the 5α- relative to 5β-metabolites of cortisol (Ulick *et al.*, 1977).

Selective glucocorticoid receptor (GR) blockade with RU 38486 (RU 486) did not decrease blood pressure as would be expected if the GR were responsible for the development of hypertension in AME. Instead, a significant increase in mean blood pressure was observed compared with the pretreatment period, indicating that the GR was not contributing to the development of hypertension.

The constellation of clinical, hormonal, and metabolic features that have been described in patients with AME including sib pairs (DiMartino-Nardi *et al.*, 1987; Shackleton *et al.*, 1985) suggests an inborn error of metabolism attributable to a defect in the gene encoding 11β-hydroxysteroid dehydrogenase (New *et al.*, 1982; Oberfield *et al.*, 1983). Attempts to evince 11-HSD deficiency in parents have yielded positive results in one father whose excretion of tritiated water was slightly low compared with controls (M. I. New, P. Speiser, and H. L. Bradlow, unpublished) and in one mother with mild hypokalemia and hypertension (Stewart *et al.*, 1988). The fact that a subtle enzyme defect could not consistently be demonstrated in parents of these patients

(Shackleton *et al.*, 1985; DiMartino-Nardi *et al.*, 1987) does not negate the genetic theory.

Apparent mineralocorticoid excess occurs in all racial groups and is equally distributed between males and females (Stewart *et al.*, 1987) (Table VIII). Among patients identified to date, ages at diagnosis have ranged from 6 months to 20 years. The fact that no adults with the condition have been described suggests that the disease, if untreated, is invariably fatal. Five patients have died, yielding a mortality rate of 25%. Most patients had some evidence of end organ damage at the time of diagnosis. Two patients had severe complications of aortic insufficiency, one requiring aortic valve replacement. Although the initial therapeutic response to mineralocorticoid blockade with spironolactone is good, patients eventually require two to three antihypertensive medications to maintain their blood pressure within a safe range. It is not well understood why the hypertension in this syndrome follows a more malignant course than in other forms of mineralocorticoid-induced hypertension.

2. *Licorice Ingestion*

Studies of licorice ingestion provide further insight into the mechanism of glucocorticoid-mediated hypertension. A decade after the first complete description of a patient with AME, Stewart, Edwards, and collegues were able to show that when healthy adult males were given 200 g/day of licorice (containing 580 mg glycyrrhizic acid, the active component of the confection) their hormonal and metabolic profiles paralleled the profile of AME patients (Stewart *et al.*, 1987). This led to the crystallization of a proposal first promulgated by New in 1982 (New *et al.*, 1982): An increase in cortisol versus metabolically inactive cortisone causes saturation of cortisol binding globulin, allowing cortisol to gain access to the mineralocorticoid receptor (MR), which shows no intrinsic preference for aldosterone as a ligand. Thus, the 11-HSD is the integral link in protecting renal MR from the normally extant 1000-fold excess physiologic concentration of cortisol compared with aldosterone (Edwards *et al.*, 1988; Funder *et al.*, 1988).

Recent evidence suggests that the metabolic effects of carbenoxolone and glycyrrhetinic acid, in contrast with their clinical effects, may differ from each other. Stewart and Edwards (1991) have shown that carbenoxolone, in contrast with glycyrrhetinic acid, did not change urinary (allo'THF + THF)/THE, or alter plasma cortisone in volunteers. The metabolic profile resembles that of a form of AME reported by Ulick.

3. *Alternative Forms of AME*

Ulick has described a Type 2 AME in which the cortisol metabolic clearance rate is delayed, but the conversion of cortisol to cortisone is not impaired (Ulick *et al.*, 1989). Unlike patients with the classic form of AME, hypertension in the Type 2 patients is ameliorated by treatment with dexamethasone (Ulick *et al.*, 1990). It has been suggested that these cases might be explained based on a generalized defect in cortisol metabolism. Alternatively, the absence of a discernible alteration in the THE:THF ratio may reflect equivalent defects in both the oxidative and the reductive components of the 11-HSD system. Support for the latter theory derives from *in vivo* studies with carbenoxolone in which cortisol half-life was prolonged, yet the THE:THF ratio was not perturbed (Stewart *et al.*, 1988).

Liddle has described a familial hypertensive syndrome with signs of mineralocorticoid excess responsive to triamterene, but not to spironolactone (Liddle *et al.*, 1963). The proposed etiology for this disorder is enhanced renal tubular sensitivity to low levels of mineralocorticoids; several additional cases have since been reported (Milora *et al.*, 1967; Hellbock and Reynolds, 1970; Wachtel *et al.*, 1975; Costin *et al.*, 1979; Wang *et al.*, 1981).

Although the syndrome of AME usually results in severe and often fatal hypertension and has most often been diagnosed in children (New and Levine, 1977; New *et al.*, 1977; Winter and McKenzie, 1977; Werder *et al.*, 1974; Fiselier *et al.*, 1982; Honour *et al.*, 1983; Harinck *et al.*, 1984; Shackleton *et al.*, 1985), one adult-onset case has been recognized (Stewart *et al.*, 1988). This patient was thought to have the classic form of AME, but unlike most of the others reported, he was responsive to dexamethasone treatment in terms of restoring positive potassium balance and elevation of plasma renin activity, although blood pressure was not significantly changed. Other classic cases where dexamethasone was tried with some salutary effects were reported by Werder *et al.* (1974) and others (Fiselier *et al.*, 1982; Honour *et al.*, 1983; Harinck *et al.*, 1984; Shackleton *et al.*, 1980). Secondary effects of apparent mineralocorticoid excess have also been reported, most notably the coexistence of large renal calculi (DiMartino-Nardi *et al.*, 1987), and in one case actual rickets due to secondary hyperparathyroidism (Batista *et al.*, 1986).

Clinical characterization of the syndrome of apparent mineralocorticoid excess has provided unique and powerful insights into the importance of 11β-hydroxysteroid dehydrogenase in blood pressure homeostasis. For the clinician, there are as yet several unresolved questions: (1) Why are these patients not Cushingoid in light of the low

TABLE VIII

REPORTED CASES OF APPARENT MINERALOCORTICOID EXCESS (AME)

Patient[a]	Patient age[b] (years)	Sex	R[c]	Blood pressure (mm Hg)	Aldosterone[d] (ng/dl)	Citation[e]
1	3	F	10.2	175/115	1.9	(1)
2D (14 years)	3	F	>7	144/104	ND	(2)
3D (12 years)	0 1/12 (2 9/12)	F	9.8	180/120	ND	(3)
4	1 7/12	M	>4	140/100	ND	(3)
5	9	M	>10	250/180	ND	(4)
6	3 3/12	F	40	125/85	—	C. Philippou (1978)/
7	2 (4)	M	15.9	140/90	1.3	(5)
8D	1 7/12	M	45	150/110	3	(6)
9D (5/12 year)	0 5/12	M	70	200/100	1.1	(7)
10	0 9/12 (19)	F	15	150/100	2.4	(8)
11R	3	F	31.2	170/110	<0.2	(9)
12R	3 9/12	F	13.4	200/129	—	(9)
13	7	F	29.8	160/120	ND	(10)
14	9	M	26.9	170/100	—	J. S. D. Winter (1988)/
15	3	M	7.5	200/110	—	Peskovitz (1986)/
16	21	M	13.5	200/145	<3.4	(11)
17R	2 (9 4/12)	F	8.9	130/90	ND	(12)
18R	2 6/12 (4 4/12)	F	20	142/98	ND	(12)
19	14 9/12	M	8	130/90	ND	(12)
20	2 3/13	M	129			Wood (1992)/

[a] D, patient died (age at death); R, the adjacent patients are siblings.

[b] Two ages are presented in some cases. The first is the one in which hypertension was reported. The second, in parenthesis, is the age at which AME was diagnosed. A single figure indicates that hypertension was found at the time of AME diagnosis.

[c] $R = (THF + 5\alpha THF)/THE$.

[d] Normal range is 5 to 20 ng/dl. ND, not detected.

[e] (1) Werder *et al.* (1975); (2) New *et al.* (1977); (3) Winter and McKenzie (1977); (4) Ulick *et al.* (1979); (5) Shackleton *et al.* (1980); (6) Fiselier *et al.* (1982); (7) Honour *et al.* (1983); (8) Harinck *et al.* (1984); (9) Shackleton *et al.* (1985); (10) Batista *et al.* (1986); (11) Stewart *et al.* (1988); (12) Monder *et al.* (1986).

/ Unpublished.

plasma ACTH and accompanying prolonged cortisol half-life? (2) Conversely, if they are not in a state of cortisol excess as reflected by low plasma ACTH levels, how are they able to survive stressful illness without cortisol supplementation? (3) What are the relationships between the variant syndromes that have been described? (4) Why is there so much heterogeneity among patients with respect to the therapeutic efficacy of low-sodium diet, spironolactone, triamterene, and dexamethasone?

4. *The Defect in AME is Mainly in the Kidney*

That the primary defect of AME was in the kidney tubule was deduced from the fact that hypertension and salt imbalance was controlled using the therapeutic regimen utilized for the treatment of aldosteronemia, despite clear evidence for hypoaldosteronemia in these patients. How the imbalance in conversion of cortisol to cortisone is related to juvenile hypertension emerges from the observation that, in all species, cortisol (and corticosterone) at sufficiently high concentrations are mineralocorticoids. Under normal physiological conditions, the active mineralocorticoid is aldosterone; glucocorticoids have no important role in salt metabolism. It is now known that MR bind aldosterone and corticosterone (or cortisol) with equally high affinity (Amelung *et al.*, 1953b). Funder (1987) posed the following significant question: how does aldosterone get its message through to the mineralocorticoid target tissues in the face of much higher circulating free levels of the glucocorticoids? In attempting to answer this question, Stewart and Edwards (1990) and Funder (1990a) have presented a refined and expanded version of earlier proposals (New *et al.*, 1982) that were designed to explain the role of 11β-dehydrogenase in blood pressure control. They proposed that the role of 11β-dehydrogenase in highly vascular tissues, such as the kidney, is to provide an enzymatic barrier to prevent the accumulation of levels of glucocorticoid that would compete with aldosterone for MR. In AME, this barrier is defective, aldosterone secretion is suppressed, and cortisol, because it cannot be inactivated, is utilized by the receptor as if it were a mineralocorticoid.

B. 11-Oxoreductase Deficiency

Independent reports by Taylor *et al.* (1984) in England and Phillipou (Phillipou and Higgins, 1985) in Australia described female patients with apparent deficiency of 11-reduction. These women presented with hirsutism and bilaterally enlarged adrenal glands. Plasma androgen concentrations were about five times above normal; plasma and urinary free cortisol were normal. Examination of the urinary steroids revealed a 7- to 9-fold increase in cortisol metabolites and a 6- to 10-fold increase in androgens. The ratio of THE/(THF + 5αTHF) was extremely high (26, normal ca. 1). These are the only recorded examples of selective 11-oxoreductase deficiency. The evidence indicates two conditions, AME and 11-oxoreductase deficiency, in which 11-HSD appears to be expressed in opposite directions with little reversibility.

VIII. Enzymology and Molecular Biology

A. The Uniqueness of 11-HSD

The metabolism of a steroid in its target cell determines its effective intracellular concentration, its accessibility to its receptor, and its ability to affect cell function. Thus, any catabolizing enzyme could qualify as a candidate for controlling tissue steroid levels. In this sense, the role of 11-HSD is potentially not different from that of any other enzyme. There are, however, characteristic properties of 11-HSD that, taken together, make it unique. First, 11-HSD affects the activity of glucocorticoids and no other steroid class. Second, 11-HSD is the dominant, if not the sole, enzyme responsible for modifying intracellular corticosteroid levels in many tissues. Third, the enzyme is reversible, enabling it to control the direction of corticosteroid metabolism, thus permitting it to catalyze 11-oxidation to diminish intracellular glucocorticoid concentrations, or 11-reduction to increase them. Fourth, in circumstances where selectivity of aldosterone activity is crucial, such as in the kidney or brain, the enzyme specifically depletes glucocorticoid, without affecting mineralocorticoid.

B. Preparation and Properties of Homogeneous 11-HSD

1. *Purification*

The selective directionality of 11-HSD catalysis has led to numerous hypotheses, some assuming a unique reversible enzyme, others a complex of separate, intercommunicating proteins expressing either 11β-dehydrogenase or 11-oxoreductase activities. Attempts to separate these activities or purify 11-HSD have, in the past, been unsuccessful (Hurlock and Talalay, 1959; Bush *et al.*, 1968). The enzyme of rat liver is embedded in the endoplasmic reticulum, and because of this, its

purification presents particular problems unique to membrane-bound proteins. Release of the protein from the membrane without denaturing it is usually achieved by displacing the detergent-like native environment with a synthetic detergent (Hjelmeland and Chrambach, 1984; Helenius and Simons, 1975; Tanford and Reynolds, 1976; Rajin, 1972; Lakshmi and Monder, 1985a). Detergent extraction releases 11-HSD in a soluble state, but does not separate oxidation and reduction activity (Lakshmi and Monder, 1985a).

To investigate the properties of 11-HSD, it was purified from rat liver using NADP–agarose affinity chromatography. The homogeneous enzyme preferentially used NADP as cosubstrate; NAD was about 30% as effective (A. Marandici and C. Monder, unpublished observations). The enzyme expressed no detectable 11-oxoreductase activity. This observation initially reinforced the conclusion that 11-HSD is a complex of separate 11β-dehydrogenase and 11-oxoreductase components (Lakshmi and Monder, 1988).

2. *Properties of Purified Enzyme*

The homogeneous 11β-dehydrogenase is a glycoprotein with a monomer molecular weight of about 34,000. It readily aggregates into clusters of 5 to 11 units, due to the mutual attraction of its hydrophobic regions. Total liver 11-HSD activity is the sum of high K_m (6 μM, corticosterone as substrate) and low K_m (90 nM) activities. Purified enzyme expresses the kinetic behavior of the high K_m form (Monder and Lakshmi, 1989b).

Kinetic analysis and ligand binding studies of purified 11-HSD reveals that the behavior of the enzyme conforms to an ordered sequential mechanism (Monder *et al.*, 1991). In the oxidative direction, the obligatory sequence of addition of cosubstrates requires that NADP be bound first, followed by corticosteroid. Because the enzyme does not express 11-oxoreductase activity, no kinetic analysis has been possible in the reductive direction.

3. *Antibodies*

Monospecific, polyclonal antibodies to homogeneous rat liver 11-HSD generated in rabbits (Monder and Lakshmi, 1990) have been used to investigate the organ-specific distribution and physiological functions of this enzyme in several organs (Monder, 1991a,b). In all tissues of the rat thus far investigated, 11-HSD antibody reveals a 34K protein indistinguishable from that of the rat liver enzyme (Monder and Lakshmi, 1990). The intensities of the bands on electrophoretograms after Western blot analysis generally corresponded in magnitude with

enzyme activity. A few tissues that expressed 11-HSD activity had no evidence of 11-HSD-like immunoreactivity, suggesting that they contain possible alternative enzyme forms (Monder, 1991a).

C. Molecular Analysis

1. *Structure–Function Predictions*

As a first step in the molecular genetic analysis of this enzyme, clones encoding 11-HSD were isolated by probing a rat liver cDNA library in the phage λgt11 with a monospecific antiserum to 11-HSD (Agarwal *et al.*, 1989). Analysis of clones demonstrated that the mRNA encoding this enzyme in the rat has an open reading frame that predicts a polypeptide of 287 residues with a molecular weight of 31,800, in contrast to the purified protein's actual MW of 34,000. The difference may be due to glycosylation; there were two potential sites for N-glycosylation in the predicted sequence. The rat clone was subsequently used to isolate human 11-HSD cDNA clones from a testis library (Tannin *et al.*, 1991). The amino acid sequence of human 11-HSD predicted from the nucleotide sequence is 79% identical to the corresponding rat sequence.

A search of sequence databases revealed that the predicted sequence of 11-HSD was related to several other prokaryotic and eukaryotic enzymes (Baker, 1989, 1990a). These include steroid 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (Marekov *et al.*, 1990), a murine 27-kDa adipocyte protein of unknown function, the *nodG* gene product of the nitrogen fixing bacterium *Rhizobium meliloti*, ribitol dehydrogenase from *Klebsiella aerogenes*, the *act III* gene product from *Streptomyces coelicolor*, human estradiol 17β-hydroxysteroid dehydrogenase, and dihydrodiol dehydrogenase from *Pseudomonas* species. Although it could not be readily aligned directly with 11-HSD, alcohol dehydrogenase of *Drosophila melanogaster* showed significant similarity to several of the other dehydrogenases used in the alignment (Baker, 1990b). Examination of these alignments (excluding *Drosophila* alcohol dehydrogenase) revealed a total of nine residues that were conserved in all proteins. These residues are likely to be structurally or functionally important. Although it, too, could not be aligned with 11-HSD using the computer algorithm, human 3β-hydroxysteroid dehydrogenase retains six of these nine residues in a similar arrangement (Fig. 4). Three of these residues are in an area near the amino terminus that is similar to known nucleotide cofactor binding sites of other enzymes, including

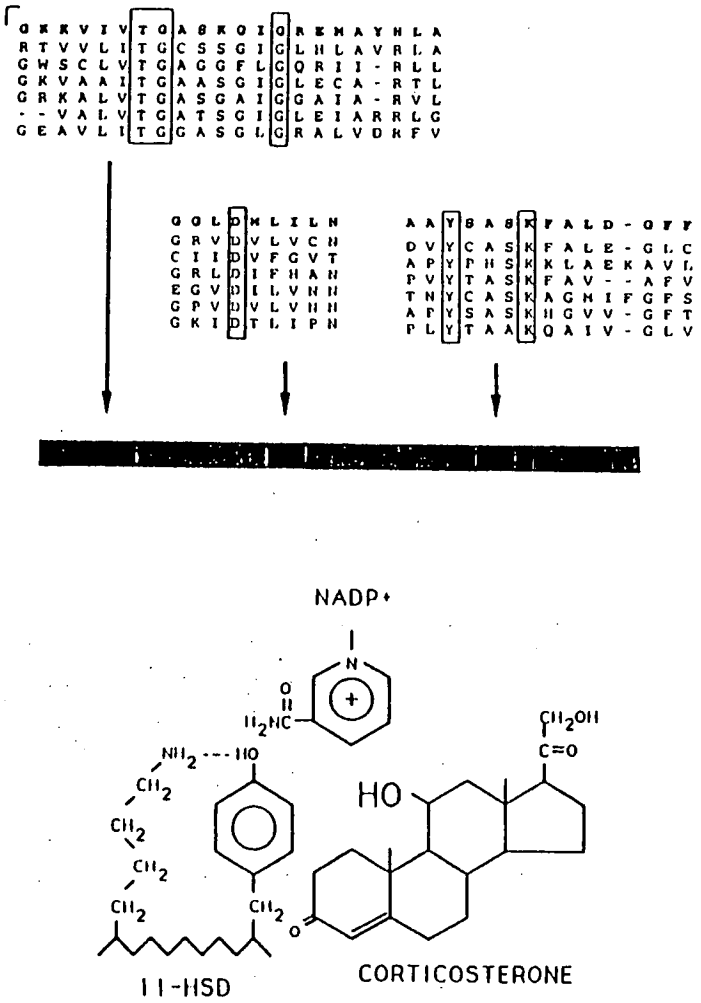

FIG. 4. (Top) Conserved amino acid sequences in 11β-hydroxysteroid dehydrogenase (bold letters) and related enzymes. In descending order, the sequences are 11-HSD, 17β-hydroxysteroid dehydrogenase, 3β-hydroxysteroid dehydrogenase, ribitol dehydrogenase (Klebsiella aerogenes), actIII protein from *Streptomyces coelicolor*, dihydrodiol dehydrogenase from *Pseudomonas* sp. Amino acids shown are A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. Absolutely conserved residues are boxed. The positions of these sequences are indicated by the dark boxes within the shaded box depicting the 11-HSD amino acid sequence. (Bottom) Proposed active site of rat liver 11-HSD showing the spatial relationships of tyrosine-179, the pyridine ring of NADP+, lysine-183, and position 11 of the steroid substrate.

yeast alcohol dehydrogenase (Jornvall *et al.*, 1981). If the three absolutely conserved residues distal to the cofactor binding site (Asp-114, Tyr-183 and Lys-187, human 11-HSD; Asp 110, Tyr-179, Lys-183, rat liver 11-HSD) participate in the catalytic function of the enzyme, they should be near the pyridine ring of NADP+ and/or the 11α position of the steroid, a hypothesis that could be tested if the three dimensional structure of 11-HSD could be determined by X-ray crystallography.

The three-dimensional structure of a related enzyme should also provide useful information concerning the functional significance of the conserved residues. Crystallographic studies of 3α,20β-hydroxysteroid dehydrogenase of *S. hydrogenans* were recently reported (Ghosh *et al.*, 1991),. In this related enzyme, the conserved region near the amino terminus does form part of the nucleotide cofactor binding site. The conserved tyrosine residue (Tyr-152 in 3α,20β-HSD) is indeed located near the pyridine ring of the cofactor in a cleft that is presumed to be the steroid binding site. The conserved lysine is directly behind the tyrosine (i.e., on the opposite side of the tyrosine ring from the cofactor). There is demonstrable bridging of electrons between the phenolic hydroxyl of tyrosine and the δ-amino group of lysine, suggesting an interaction between these groups. These findings support the idea that the conserved tyrosine and lysine participate in the catalytic function of the enzyme by facilitating the transfer of a hydride radical from the steroid to the cofactor. In contrast, the conserved aspartate (Asp-82 in 3α,20β-HSD) is not located near the cofactor or the steroid, and its functional significance is difficult to assess from these studies. Thus, it will be necessary to test the importance of these residues in 11-HSD by *in vitro* mutagenesis and expression of the cDNA in cultured cells.

2. *Functional Characteristics of Recombinant 11-HSD*

To determine whether both 11β-dehydrogenase and 11-oxoreductase activities resided in the same enzyme, a full-length cDNA clone was expressed in Chinese hamster ovary (CHO) cells by transient transfection with a plasmid expression vector. Enzymatic activities were determined by incubating transfected cells with radioactive substrates. Whereas normal CHO cells did not contain significant 11β-dehydro-

genase and 11-oxoreductase activities, these cells developed roughly equal levels of both activities (about 40% conversion of substrate to product after 20 h) after transfection with the expression plasmid. Addition of glycyrrhetinic acid, a known inhibitor of 11β-dehydrogenase, reduced expressed dehydrogenase activity by 50% without affecting reductase activity (Lakshmi and Monder, 1985b).

To obtain kinetic parameters for the two activities, 11-HSD was expressed at higher levels using recombinant vaccinia virus (Agarwal *et al.*, 1990). Dehydrogenase and reductase activities were assessed in cellular lysates in the presence of saturating concentrations of NADP and NADPH, respectively. At pH 7.0, the recombinant enzyme had very similar K_m and first-order rate constants (V_{max}/K_m) for both activities. These results were consistent with the hypothesis that both dehydrogenase and reductase activities reside in a single enzyme. Exposure to NADP resulted in rapid and irreversible inactivation of the reductase activity of the enzyme, a phenomenon consistent with the instability of the reductase during attempted purification from rat liver.

In contrast, when the recombinant enzyme was prepared from cells grown in the presence of A_1 tunicamycin (an inhibitor of glycosylation), dehydrogenase activity was reduced by about 50%, whereas reductase activity was unaffected. This was associated with increased amounts of a 31-kDa enzyme species that presumably represented the unglycosylated enzyme. This suggests that the dehydrogenase activity of the enzyme may depend on adequate glycosylation.

3. *Tissue Distribution of 11-HSD Expression*

In initial studies, the rat cDNA clone hybridized to a single mRNA species of approximately 1600–1700 nucleotides in samples from testis (highest), liver, kidney, and lung but did not hybridize to samples from heart or colon. This distribution roughly paralleled that of 11β-dehydrogenase activity.

A subsequent study (Krozowski *et al.*, 1990) suggested that the rat kidney actually contains several cross-hybridizing mRNA species of 1900, 1600, and 1500 nucleotides (renal cortex/medulla) and 1700 nucleotides (renal papilla). In this study, the highest level of expression was found in the liver, followed respectively by kidney, lung, testis, hippocampus, heart, and colon.

In further studies of expression in rat brain (Moisin *et al.*, 1990a,b), an apparently identical mRNA species was found in all areas, but at highest levels in the hippocampus and cortex. It is speculated that 11-HSD regulates the access of glucocorticoids to cerebral mineralocorticoid and/or glucocorticoid receptors, thus modulating steroid hormone effects of cerebral function.

The tissue distribution of the human mRNA differs from that in the rat; it is expressed at very high levels in the liver and at much lower levels in the kidney. The significance of these findings, given the importance of this enzyme activity in the kidney, is not yet clear, but it is consistent with the idea that there may be additional proteins with 11-HSD activity in the kidney.

4. *Genetic Analysis of Human 11-HSD*

To determine the chromosomal location of the human 11β-hydroxysteroid dehydrogenase (*HSDB11*) gene, a cDNA clone was hybridized to DNA samples from a panel of human–rodent somatic cell hybrid lines. Hybridization to human-specific bands was consistent with a location on chromosome 1 (Tannin *et al.*, 1991).

Hybridization of blots of uncloned human genomic DNA that had been digested with restriction endonuclease *Hind*III demonstrated that there was a single *HSD11* gene that was carried on two fragments. Sequence analysis of these fragments showed that they carried a single gene consisting of six exons, the first four of which were contained on the smaller fragment. Comparison of the maps of restriction sites in these fragments with results of hybridization to uncloned DNA revealed that there must be an additional *Hind*III fragment(s) of undetermined size in intron 4 that contains *Eco*RI and *Bam*HI sites.

a. Transcriptional Regulation of the HSD11 Gene. Primer extension analysis indicated that transcription of the human *HSD11* gene starts 93 bp upstream from the start of translation (Tannin *et al.*, 1991). This yields a 5′ untranslated region very similar in length to that of rat 11-HSD mRNA. There is no TATA box in the 5′ flanking region, but there is a consensus CAAT box (CCAATC) 76 bases upstream from the start of transcription. An 8-bp palindromic sequence (CTGTACAG) was present 188 bp upstream from the start of transcription. It resembles part of a glucocorticoid response element (Evans, 1988), which would be consistent with the known ability of glucocorticoids to increase levels of 11-HSD activity. However, its functional significance requires further study, particularly in light of recent work suggesting that glucocorticoids do not alter the level of *HSD11* gene expression in rat liver, lung, or kidney (Krozowski *et al.*, 1990).

Recent S1 nuclease analysis suggests that the different-sized mRNA transcripts observed in rat kidney apparently have different 5′ extensions. Cloning studies suggested that some transcripts have a divergent 5′ coding sequence that encodes a putative protein with a

truncated amino terminus (Krozowski *et al.*, 1992). Comparison of the sequence of the truncated clones with that of the human gene suggests that these clones originate by transcription within the first intron of the corresponding rat gene. It is not yet known whether the putative protein is functional or even whether it is synthesized *in vivo*.

b. Possibility of Additional 11-HSD Enzymes. In addition to the putative truncated form of the protein, other evidence suggests that there may be an additional enzyme(s) with 11-HSD activity. As mentioned, there appears to be some discrepancy between the levels of 11-HSD activity and *HSD11* mRNA in human kidney. In rat kidney, anti-11-HSD sera react with proximal tubules but not with distal tubules/collecting ducts, although the latter represent the main site of mineralocorticoid action (Rundle *et al.*, 1989a). The 11-HSD activity of isolated rabbit distal tubules and collecting ducts differs markedly in kinetic properties from the enzyme in the proximal tubule (which is similar or identical to the enzyme in the liver) in having a K_m about 100-fold lower (Naray-Fejes-Toth *et al.*, 1990). A similar low K_m form has been detected as a minor species in the liver (Monder and Lakshmi, 1989b). Furthermore, histochemical studies have suggested that the distal tubule contains an 11-HSD activity that requires NAD rather than NADP (Mercer and Krozowski, 1992). No mRNA was detected in heart, yet heart readily converts cortisol to cortisone (C. Monder and A. Marandici, unpublished observations) (Kolanowski *et al.*, 1981). Because Southern blotting studies indicate that rats and humans carry only one *HSD11* gene, the gene(s) encoding any additional 11-HSD activities must be sufficiently different from *HSD11* in their nucleotide sequences that they do not cross-hybridize.

A number of questions regarding the functions of 11-HSD may be answered by molecular genetic analysis of patients with inherited enzymatic deficiencies. Because both dehydrogenase and reductase activities apparently reside in the same enzyme, it will be of obvious interest to search for mutations in the HSD11 gene(s) associated with AME and 11-oxoreductase deficiencies and correlate their effects on enzymatic function with clinical phenotype.

IX. 11-HSD Function in Specific Organs

A. Kidney

1. *Mineralocorticoid Receptors and 11-HSD*

We have discussed the fact that the characteristic biochemical abnormality of AME is a severe loss in the ability of patients with this disability to oxidize cortisol. A working model connecting 11-HSD activity and blood pressure control evolved from the convergent findings of many laboratories. It was discovered that, *in vitro*, the renal MR bound aldosterone and the glucocorticoids, corticosterone and cortisol, with comparable affinity (Krozowski and Funder, 1983; Arriza *et al.*, 1987; Armanini *et al.*, 1985). That this behavior is an intrinsic property of MR was shown by Arriza *et al.* (1987) using cloned recombinant MR derived from placental cDNA expressed in COS cells. However, *in vivo*, aldosterone was selectively taken up by the MR of some tissues, such as kidney, parotid, and colon (Sheppard and Funder, 1987a,b), to the exclusion of glucocorticoids. That these tissues are aldosterone selective appeared to be in conflict with the *in vitro* data. To reconcile the *in vitro* and *in vivo* evidence, a hypothesis to explain steroid selectivity was developed based on the observation that glucocorticoids are uniquely sequestered to corticosteroid binding globulin (CBG) and are thus made unavailable to MR (Krozowski and Funder, 1983; Sheppard and Funder, 1987a). It was, however, found that aldosterone selectivity persisted *in vivo* in young rats with little or no circulating CBG (Sheppard and Funder, 1987a,b). Since MR is coded for by a single gene (Arriza *et al.*, 1987), it was considered unlikely that selectivity depends on tissue- or age-specific variations in its intrinsic properties. However, hormone-dependent gene regulation by MR showed a preference of aldosterone over cortisol (Arriza, 1991). It was recently suggested that post-translational modifications of MR may play a role in mineralocorticoid binding and specificity, but this possibility remains to be evaluated (Doyle *et al.*, 1988). A search for specific regions of MR localization in the rat nephron revealed that MR are not distributed uniformly throughout the renal tubule and are localized to the distal tubule. Krozowski *et al.* (1989), using an antiserum corresponding to the hinge region of human MR (Arriza *et al.*, 1987), showed that MR are localized in the principal cells of the cortical collecting duct. MR were not detected in the proximal tubule and glomerulus (Wrange and Yu, 1983). They have been reported to be present in the distal convoluted tubule and the thick ascending limb of the loop of Henle (Doucet and Katz, 1981; Katz, 1990; Farman *et al.*, 1983). Thus, although there is some uncertainty about the range of MR distribution in the distal tubule, its localization to this region is not questioned (Krozowski *et al.*, 1989; Rundle *et al.*, 1989b; Bonvalet, 1991).

The renal distribution of immunoreactive 11-HSD and MR was very different. Polyclonal antibodies (Edwards *et al.*, 1988; Rundle *et al.*, 1989a) and monoclonal antibodies (Castello *et al.*, 1989) to 11-HSD revealed specific immunoreactive staining in the proximal tubules of the inner cortex. The focal distribution of 11-HSD and MR within the

tubule suggested a model in which blood filtrate containing aldosterone and a thousandfold greater concentration of corticosterone must pass through a region of high 11-DH activity, located in the proximal tubule and vasa recta. During this passage corticosterone is completely inactivated, leaving the aldosterone, which is not a substrate of the enzyme, unaltered, permitting its binding to MR to occur unopposed. Since 11-DH and MR do not colocalize, a paracrine relationship between them is inferred. An explanation of the consequences of the deficiency of 11-DH in humans is predicated on the assumption that these structural and functional relationships apply to human as well as to rat kidney.

2. *Role of Glucocorticoid Receptors*

The hypothesis based on this model assumes that all available glucocorticoid is oxidatively inactivated as it passes through the region of high 11-HSD activity. The model in this form proved to be extremely useful and enabled puzzling aspects of AME to be explained. It is, however, known that the renal tubule contains glucocorticoid receptors. These mediate glucocorticoid-specific effects on the kidney including effects on renal hemodynamics, acid and water excretion, gluconeogenesis, and sodium–potassium ATPase (Katz, 1990; Kinsella, 1990). Consequently, complete inactivation of corticosterone or cortisol would not be a desirable option for the kidney. Therefore, the hypothesis originally proposed that envisioned a relationship between the MR and 11-HSD that excludes GR-mediated effects must be revised. Glucocorticoid receptors are known to be distributed along the nephron (Farman *et al.*, 1991; Katz, 1990). The number of glucocorticoid binding sites in the thick ascending limb of the loop of Henle, for example, is 100-fold higher than the aldosterone binding sites (Lee *et al.*, 1983). Maximal binding capacity of the cortical collecting tubule for corticosterone is greater than for aldosterone (Katz, 1990). Thus, though glucocorticoids at moderate concentrations may mediate renal function by way of MR, GR are important, as well (Naray-Fejes-Toth and Fejes-Toth, 1990; Clore *et al.*, 1988). The emerging concept that 11-HSD plays an important role in mediating GR dependent processes is supported by the observation that there is a strong correlation between GR and 11-HSD distribution in tissues (Whorwood *et al.*, 1992).

3. *The Protector Role of 11-HSD: Modifying the Hypothesis*

The hypothesis that renal 11-HSD enables MR to interact selectively with aldosterone as both ligand and effector by inactivating potentially competing glucocorticoids was supported by extensive laboratory

and clinical data (Funder, 1990a,b; Stewart and Edwards, 1990; Edwards *et al.*, 1989; Monder and Shackleton, 1984; Monder, 1991b). However, the proximal 11-HSD-distal MR model initially proposed to explain the protector function of 11-HSD proved to be less and less adequate as it was reexamined. The great physical distance between 11-HSD and MR appeared to result in an inefficient functional unit. Furthermore, that all available corticosteroid must be inactivated by 11-HSD, implicit in the model, was an untested assumption. The functions of renal GR and the possibility of a functional link between active (steroid bound) GR and the expression of MR remained to be investigated.

The requirement of the original model that access of aldosterone to MR could be achieved only if the tubular filtrate were completely cleansed of glucocorticoid was an extremely stringent one. In order to accommodate this requirement, it was proposed that 11-HSD is strategically distributed along the nephron in order to oxidize residual glucocorticoid. Bonvalet *et al.* (1990) found 11-HSD activity in the distal as well as the proximal portions of the rabbit kidney tubule. Possibly, it was suggested, 11-HSD and MR may coexist in distal cells (Naray-Fejes-Toth *et al.*, 1991; Stewart *et al.*, 1991; Bonvalet, 1991). Gradient fractionation of rat kidney tubules indicated that 11-HSD was indeed in the distal as well as proximal regions (Edwards *et al.*, 1988). The immunohistochemical studies that led to the conclusion that 11-HSD was localized solely in the proximal region of the nephron were in obvious conflict with the enzyme activity data (Rundle *et al.*, 1989a; Castello *et al.*, 1989). The lack of immunoreaction of the distal tubular enzyme with rat liver 11-HSD antibody could be explained by assuming that the enzyme was not easily accessible to antibody because of a transcellular barrier, or was a distantly related antigen. Naray-Fejes-Toth *et al.* (Naray-Fejes-Toth and Fejes-Toth, 1990; Bonvalet *et al.*, 1990), using rabbit kidney cortical collecting tubules isolated by solid-phase immunoadsorption, conclusively showed that there was 11-HSD activity in this region that did not react with antibody on Western blot analysis.

Naray-Fejes-Toth *et al.* (1991) found that the level of 11-HSD activity in monolayer preparations of CCD cells was sufficient to completely convert corticosterone to 11-dehydrocorticosterone in a single pass through the membrane. Therefore, in an individual MR-containing cell, there may be enough 11-HSD to inactivate glucocorticoid completely, thus satisfying the requirement that aldosterone bind MR unencumbered by competing steroids. Differences were also noted in the distribution of 11-HSD in the renal cortex and medulla

(Castello *et al.*, 1989). Consistent with the above postulated autocrine role, the 11-HSD of the distal tubule was more active than the proximal tubule (Edwards *et al.*, 1988; Castello *et al.*, 1989; Bonvalet *et al.*, 1990). A schematic view of the current understanding of corticosteroid associated interactions in normal kidney is shown in Fig. 5.

Evidence that salt metabolism may be mediated through GR as well as MR have been presented by Naray-Fejes-Toth and Fejes-Toth (1990) and Funder *et al.* (1990). The following observations support this conclusion: (*a*) AME patients are more sensitive to cortisol than aldosterone in terms of increased blood pressure and sodium retention; (*b*) in pseudohypoaldosteronism, a condition characterized by low or no MR, the electrolyte effect of cortisol results in part from occupancy of GR; (c) RU 28362, a GR-specific glucocorticoid that does not bind MR, affects electrolyte excretion via GR; (*d*) RU 28318, a specific MR antagonist, does not diminish the electrolyte effect of RU 28362; (*e*)

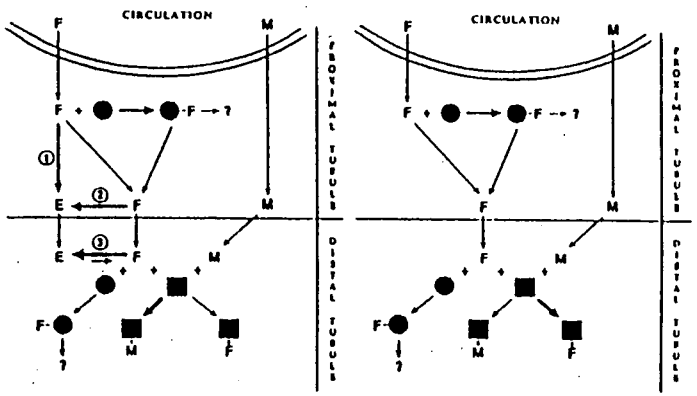

FIG. 5. A current view of corticosteroid associated interactions in normal and 11-HSD-deficient kidney. Heavy arrows indicate dominant pathways. (Left) Normal kidney. Cortisol (F) in the proximal (and possibly distal) tubule mediates glucocorticoid dependent events via glucocorticoid receptor (GR). (●) The level of F available to GR is mediated by 11-HSD (1). Steroid dissociated from GR is oxidized to cortisone (E) (2) to prevent its reentry into the system. F entering from the proximal tubule and other sources may compete with aldosterone (M), for mineralocorticoid receptor (MR). (■) This competition is prevented by oxidation of F to E (3) in the distal tubule and cortical collecting duct. (Right) 11-HSD-deficient kidney. In the absence of functioning 11-HSD, cortisol cannot be oxidized and accumulates, preferentially binding to MR, displacing M, and initiating a sequence of aldosterone-mimetic events.

immunodissected rabbit cortical connecting tubule cells responded similarly to aldosterone, dexamethasone and RU 28362; (*f*) The glucocorticoid receptor antagonist RU 486 blocked the effect of RU 28362, but the MR antagonist ZK 91587 did not; (*g*) kaluresis caused by cortisol is blocked by RU 486 (Clore *et al.*, 1988). Localization of 11-HSD mRNA by *in situ* hybridization using a cRNA probe (Agarwal *et al.*, 1989) indicated its location in the proximal tubules and in the cortical and medullary collecting tubules, a finding that accords with the enzyme distribution studies. The presence of multiple 11-HSD mRNA species in kidney is consistent with the possibility of a heterogeneous population of 11-HSD proteins that may be generated from them, some of which may be recognized by 11-HSD antibody (Krozowski *et al.*, 1990). These results also indicate that the variant forms of 11-HSD that have been proposed may be generally similar in structure.

4. *Licorice, Hypertension, and Kidney Function*

a. The Active Agent of Licorice. Valuable evidence supporting the role of 11-HSD in kidney function emerged from studies on the pharmacological behavior of licorice, a flavoring agent extracted from the roots of *Glycyrrhiza glabra*. Licorice has been used as a medicine and condiment for at least 5000 years (Davis and Morris, 1991). Glycyrrhetinic acid (GA), its active ingredient, is a cyclic triterpene whose fused ring structure, illustrated in Fig. 6, closely resembles that of the glucocorticoids. A synthetic agent developed for the treatment for gastric and duodenal ulcers, carbenoxolone (CA), is the 3-*O*-β-carboxypropionyl ester of glycyrrhetinic acid. Ingestion of either GA or CA causes

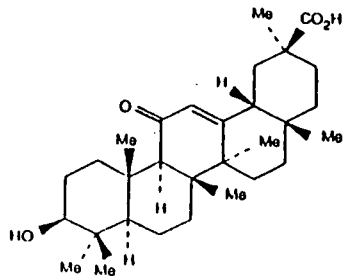

FIG. 6. Glycyrrhetinic acid (GA).

clinical effects that resemble those of aldosterone excess, including hypertension, hypokalemia, edema, polyuria, polydipsia, heart failure, and muscle weakness (Pinder *et al.*, 1976; Baron, 1983; Werning *et al.*, 1971).

b. Possible Explanation of Licorice Actions. Reevers (1948) first documented the aldosterone mimetic behavior of GA. Explanations for its properties have included suggestions that it (*a*) stimulates aldosterone production; (*b*) displaces aldosterone from nonspecific binding sites, thus increasing its effective concentration (Humphrey *et al.*, 1979; Porter, 1970); (*c*) demonstrates intrinsic mineralocorticoid activity; or (*d*) potentiates the effects of aldosterone (Humphrey *et al.*, 1979; Armanini *et al.*, 1989b). All except the last two are unlikely mechanisms (Hausmann and Tarnoky, 1968; Porter, 1970). Evidence for binding of glycyrrhetinic acid to kidney mineralocorticoid receptors was presented by Ulmann *et al.* (1975), Armanini *et al.* (1983), Takeda *et al.* (1987), and Hayashi *et al.* (1984). There is general agreement that binding of GA to MR is about 1/10,000 that of aldosterone based on competitive binding studies. It is unclear whether significant binding to human or rat kidney MR occurs even under the conditions of massive GA or CA intake. In a toad bladder model, 2.5×10^{-6} *M* CA had no effect on short-circuit current over 360 min of exposure (Gaeggeler *et al.*, 1989). Humphrey *et al.* (1979) found that CA did not displace [^{3}H]aldosterone from rat kidney nuclei. The reason may be, as Armanini *et al.* (1989a) suggest, that binding of CA to MR requires its prior hydrolysis to GA.

It has been estimated that subjects consuming 100 to 200 g of licorice per day have total circulating plasma GA levels of 80 to 480 ng/ml (Hughes and Cowles, 1977; Stewart *et al.*, 1987). The concentration of free circulating GA is lower, since 95% of GA is bound to plasma proteins (Ishida *et al.*, 1988). Thus, the concentration of GA potentially accessible to MR is too low to measurably bind to the receptor under physiological conditions. It is, however, theoretically possible that specific ligand–receptor interaction may lead to some responses resembling that of the binding of mineralocorticoid. The availability of radioactive glycyrrhetinic acid (Kanaoka *et al.*, 1988) should make it possible to determine whether its interaction with MR leads to nuclear translocation.

Additional evidence that cannot currently be reconciled with the postulated mineralocorticoid-mimetic behavior of GA is the observation that the effectiveness of GA is abolished in adrenalectomized rodents (Card *et al.*, 1953; Girerd *et al.*, 1960) and humans (Borst *et al.*, 1953; Elmadjian *et al.*, 1956) and is restored when glucocorticoids are administered (Borst *et al.*, 1953). The results indicate that a secretory

product of the adrenal cortex is an essential participant of GA action. Normal individuals ingesting glycyrrhetinic acid under controlled conditions for brief periods of time (3–10 days) showed significant decrease in cortisol oxidation to 11-oxo metabolites (MacKenzie *et al.*, 1990), a finding consistent with an inhibitory effect on 11-HSD (Mattingly *et al.*, 1970; Chen *et al.*, 1990; Ojima *et al.*, 1990).

c. Glycyrrhetinic Acid and Other Inhibitors of Renal 11-HSD. Other agents affect the activity of renal 11-HSD. The inhibition of 11-HSD by gossypol, a potential male contraceptive agent extracted from cottonseed oil, resembles that of GA and CA. This observation has led to the suggestion that the hypokalemia observed in men taking this agent has the same cause as that of men ingesting licorice (Sang *et al.*, 1991). Hierholzer and co-workers (1990b) have found that bile acids, though of low inhibitory potency, are present in the human circulation at concentrations that indicate that they have the potential to modulate 11-HSD activity.

Touitou *et al.* (1984) have made the surprising observation that trilostane, a cyanoketone derivative known to inhibit 3β-hydroxysteroid dehydrogenase, increased 11β-hydroxy oxidation in sheep liver homogenates, a phenomenon that may be species specific. Perschel *et al.* (1991) found that pooled rabbit bile at low concentrations increased rat renal 11-HSD. Whether these examples represent stimulation of 11-HSD, as the authors suggest, or an expression of the ability of $P450_{11\beta}$ to catalyze the oxidation of cortisol to cortisone (Suhara *et al.*, 1986) remains undecided.

In a recent study, GA and CA were found to be extremely potent inhibitors of 11-HSD in isolated rat kidney microsomes, with K_i values of 3 n*M* (Monder *et al.*, 1989). In the range 1 to 20 n*M*, reductase was inhibited poorly (Monder *et al.*, 1989; Hierholzer *et al.*, 1991). Glycyrrhetinic acid is the most powerful known inhibitor of 11-HSD (Monder *et al.*, 1989), but it is 10-fold less potent in intact cells. The basis for this difference is unknown. A transmembrane barrier to GA or sequestration to proteins and other macromolecules has been suggested (Monder, 1991c).

The toad bladder, the amphibian counterpart of the nephron, has proven to be a useful model for studying the pharmacological action of CA and GA on the kidney tubule. Using this system, Gaeggeler *et al.* (1989) and Brem *et al.* (1989, 1991) have shown that CA allows corticosterone to be as potent as aldosterone in eliciting the mineralocorticoid response, in accord with the proposed role of CA as an inhibitor of 11-HSD.

d. Glycyrrhetinic Acid: An Inhibitor of Broad Specificity. It is note-

worthy that Morris *et al.* have shown that the metabolism of aldosterone is slowed by glycyrrhetinic acid, a potent inhibitor of cytosolic 5β-reductase and microsomal 3β-hydroxysteroid dehydrogenase (Latif *et al.*, 1990; Tamura *et al.*, 1979; Yoshida *et al.*, 1992). By slowing the rate of inactivation of aldosterone and 11-deoxycorticosterone, these agents potentiate the activity of mineralocorticoids. The two-pronged effect of GA and CA on mineralocorticoid and 11β-hydroxysteroid metabolism would therefore provide a mechanism for inactivating glucocorticoids and simultaneously enhancing the activity of mineralocorticoids.

There are other ways in which GA or CA can affect renal function (Monder, 1991c). Indirect evidence suggests that GA may inhibit glucuronide formation, since it increases the proportion of unconjugated cortisol in urine of people given massive doses of licorice (equivalent to 0.7 to 1.4 g of GA per day for 1–4 weeks) (Epstein *et al.*, 1978). A possible direct effect of glycyrrhetinic acid on ($Na^+ - K^+$) ATPase (Itoh *et al.*, 1989; Baron and Greene, 1986) may account for some of the effects of GA on the kidneys of adrenalectomized animals. The combined effects of GA on glucocorticoid oxidation at C-11, A-ring reduction, and excretion of unconjugated steroids bear a striking resemblance to the metabolic changes characteristic of AME (Monder *et al.*, 1986). The possibility that an endogenous glycyrrhetinic acid-like compound contributes to the pathology of AME cannot be excluded.

B. The Vascular Bed

It has been known for about 50 years that adrenocortical hormones influence the behavior of the peripheral blood vessels (Swingle and Remington, 1944). These influences include alterations in intra- and extracellular levels of Na^+ and K^+ critical for maintaining vascular tone (Zweifach *et al.*, 1953), and maintenance of the sensitivity of the peripheral vasculature to pressor agents (Darlington *et al.*, 1989; Grunfeld and Eloy, 1987; Ashton and Cook, 1952). These and other effects (Moura and Worcel, 1984; Nichols *et al.*, 1983, 1984; Jazayeri and Meyer, 1988; Haigh and Jones, 1990; Yasunari *et al.*, 1989) are mediated by MR and GR in vascular smooth muscle (VSM) cells. The presence of MR and GR in vascular smooth muscle provides evidence of direct action of corticosteroids on the arterial wall affecting muscle tone and responsiveness to humoral and neurogenic vasoconstrictive stimuli (Kornel *et al.*, 1975; Onoyama *et al.*, 1979).

The whole arterial tree appears to be a target organ for both mineralocorticoids and glucocorticoids (Kornel *et al.*, 1982). There is evidence that the effects of both steroid classes on vascular tissue proceed by

independent processes (Jazayeri and Meyer, 1988, 1989). Vascular smooth muscle cells in culture are affected differently by mineralocorticoid and glucocorticoids. The glucocorticoid effects are blocked by RU 486, indicating GR dependence (Kornel, 1988; Nichols *et al.*, 1985; Meyer and Nichols, 1981). High levels of glucocorticoids could, by binding both the MR and GR, contribute to the pathogenesis of essential hypertension by stimulating vasoconstriction. Funder *et al.* (1989) found that MR of the mesenteric vascular arcade is aldosterone specific *in vivo*. They suggested that, as in the kidney, 11-HSD may mediate the selective mineralocorticoid response. Funder *et al.* (1989) and Walker *et al.* (1991) have confirmed the original report of Kornel *et al.* (1982) that the vessels of the circulatory system express 11-HSD activity.

The enzyme appears to be predominantly in the smaller vessels, a finding that has been interpreted to indicate that by catalyzing the reversible inactivation of glucocorticoids, it modulates tone in the peripheral resistance beds and thereby influences blood pressure. Alternately, as occurs in the brain (see later), the availability of NADP may affect 11-HSD activity. There appears to be insufficient NADP in VSM cells to fully activate the the available 11-HSD, thus making the nucleotide a limiting factor in the expression of enzyme activity. Consistent with the proposed role of 11-HSD, the enzyme and VSM corticosteroid receptors are colocalized, indicating that the regulation of GR–steroid interaction occurs by an autocrine mechanism.

C. The Skin

The modulation of corticosteroid effects by 11-HSD appears to extend to the superficial peripheral vessels. The potency of topical corticosteroid on suppression of the inflammatory response is determined in part by its local persistence; oxidative inactivation by dermal 11-HSD diminishes its effectiveness. It has been proposed that the vasoconstrictor action of corticosteroid contributes to the potentiation of its action, by preventing its loss. Thus, dermal 11-HSD, which accelerates the destruction of inactivation of the steroid in skin, would diminish its topical effectiveness. Consequently, inhibition of 11-HSD activity in target tissues should potentiate the local action of glucocorticoids.

This concept has been put to the test by Teelucksingh *et al.* (1990) who investigated the activity of hydrocortisone on skin. Topical application of glycyrrhetinic acid inhibited dermal 11-HSD, reducing inactivation of cortisol by skin, prolonging and enhancing its topical anti-inflammatory activity. It has been proposed that this property of GA and CA explains their beneficial effects in inflammatory cut-

aneous disorders (Colin-Jones, 1957). However, recent studies have shown that 11-oxoreductase exceeds 11β-dehydrogenase activity in human skin fibroblasts. Whether this is due to the intrinsic character of the skin enzyme or to another rate-limiting step, such as lack of pyridine nucleotide (as is found in brain), is not known. These observations suggest that in human skin the preferred direction of corticosteroid metabolism is reductive (Hammami and Siiteri, 1991; Monder *et al.*, 1986) and, therefore, that the anti-inflammatory effects of topical application of GA on human skin cannot be fully explained by the inhibition of 11-HSD.

D. The Nervous System

1. *Neural 11-HSD*

Interest in the metabolism of corticosteroids in brain and pituitary evolved simultaneously with the recognition of the importance of steroids on brain function (Woodbury, 1958), on the one hand, and the importance of neuroendocrine influences on steroid secretion, on the other. Soon after cortisol had been isolated from human nerve tissue (Touchstone *et al.*, 1963), evidence for the oxidation of corticosteroids to 11-dehydrocorticosteroids by brain tissue was obtained for rat (Peterson *et al.*, 1965; Sholiton *et al.*, 1965), mouse (Grosser, 1966; Tye and Burton, 1980), dog (Miyabo *et al.*, 1973; Eik-Nes and Brizzee, 1965), and primate (Grosser and Axelrod, 1968). Despite the fact that the presence of 11-HSD in nervous tissue had been known for many years, its possible function in the central nervous system has only recently come under investigation. The working assumption is that brain 11-HSD plays an important role in the expression of glucocorticoid-dependent processes.

2. *Receptor-Mediated Selectivity of Corticosteroid Effects*

As with kidney, central MR and GR mediate corticosteroid-specific effects. Neural MR, with properties identical to those of the renal mineralocorticoid binder (Tashima *et al.*, 1989), interacts with corticosterone (or cortisol) and aldosterone with comparable affinity, and binds dexamethasone, a synthetic glucocorticoid, much less efficiently (Beaumont and Fanestil, 1983; Krozowski and Funder, 1983; Wrange and Yu, 1983). The classic glucocorticoid receptor also uses corticosterone as ligand, but prefers dexamethasone. The equivalent affinity of MR for corticosterone and aldosterone in the rat brain contrasts sharply with the clear preference of the receptor for aldosterone

in the kidney. It therefore follows that the overwhelmingly greater concentration of corticosterone (10^2- to 10^3-fold that of aldosterone) in the circulation of the rat would result in MR saturated with and largely dependent for its activity on the circulating corticosterone. The system would thus be insensitive to aldosterone, leading to the conclusion that aldosterone can have no effect on brain function.

There is strong evidence for selective aldosterone effects in the central nervous system. It is known that rat brain takes up both aldosterone and corticosterone, with a similar regional distribution. Highest uptake occurs in the hippocampus, septum, and amygdala (Birmingham *et al.*, 1979; Gerlach and McEwen, 1972; Moguilewsky and Raynaud, 1980). The analysis of brain receptor distribution using RU 26988 (a pure glucocorticoid) and RU 28318 and RU 26752 (pure antimineralocorticoids) (Coirini *et al.*, 1985) has led to the conclusion that there are only the two receptor subtypes, MR and GR. These are distributed in neurons and glial cells (Bohn *et al.*, 1991). Despite the 100- to 1000-fold excess of corticosterone over aldosterone in the circulation (Eilers and Peterson, 1964), MR receptors recognize aldosterone in the presence of corticosterone in signaling changes in salt balance, and this effect persists at physiological levels of both mineralocorticoid and glucocorticoid (McEwen *et al.*, 1986; Forman and Mulrow, 1973; Fregly and Rowland, 1985).

Intracerebroventricular (ICV) administration of aldosterone to heminephrectomized rats caused elevated blood pressure. The effect was blocked by the mineralocorticoid receptor antagonist RU 28318. Corticosterone could not replace aldosterone, nor could systemic administration of steroids reproduce these effects (Gomez-Sanchez, 1991). The hypertension induced by aldosterone administered ICV requires corticosterone, for the effect is prevented in bilaterally adrenalectomized rats, and restored by exogenous corticosterone (Gomez-Sanchez, 1991). Arriza and Evans found in a cotransfection assay that MR was more sensitive to mineralocorticoid than to glucocorticoids, despite equivalent binding affinities (Arriza *et al.*, 1988). It has been reported that corticosteroids differentially modulate nutrient intake in rats through central receptor-mediated processes. Implant of aldosterone in the paraventricular nucleus of adrenalectomized rats stimulated ingestion of fat; corticosterone stimulated carbohydrate intake (Tempel and Liebowitz, 1989).

The interrelationships between GR, MR, and corticosteroids in the central nervous system are complex. Receptor specificity varies in ways that are not immediately obvious. In early studies, the differential binding of corticosterone to receptors suggested that there may be

three receptor types in the nervous system: the classical GR and MR, and a corticosterone binding subset of MR termed CR. Binding studies with corticosterone and aldosterone *in vitro* showed no distinction between CR and MR, and the former term was abandoned, since its retention obscured the question of the specificity-conferring mechanism. To illustrate this point, MR in the circumventricular region is aldosterone selective; MR in the neurons of the limbic region is corticosterone selective. This selectivity is reflected throughout the nervous system and shows up as differential retention of corticosterone and aldosterone in different subregions.

a. Antagonistic and Synergistic Mechanisms. DeKloet and co-workers have developed a functional rationale for the preferential binding of glucocorticoids to both GR and MR under normal physiological conditions (de Kloet and Reul, 1987). They have shown that the circulating concentration of corticosterone results in 80 to 90% occupancy of cerebral MR. This generates a baseline level of continuously activated receptor that serves to monitor and interpret the animal's external environment. At basal levels of circulating corticosterone, specifically at the diurnal trough, the levels of occupancy of GR is low; under stress, or to a lesser extent at the diurnal peak, the level of circulating corticosterone increases, leading to GR occupancy, generating a negative feedback on stress-activated brain mechanisms. There are, thus, reciprocal balancing tonic-activating actions and feedback-damping mechanisms. This continuum reflects corticosteroid concentration, and the sequential, selective occupancy of corticosterone to MR and GR. Evans and Arriza (Arriza *et al.*, 1988) have suggested that MR and GR act as a binary response system for corticosterone. Their model depends on the coordinated synergistic interaction of MR and GR with overlapping sets of genes, the magnitude of the response being dependent on the circulating glucocorticoid levels. This model may be compared with the coordinated antagonistic MR- and GR-mediated effects proposed by DeKloet. Van den Berg *et al.* (1990) suggest that central MR and GR mediate opposing effects of glucocorticoids and mineralocorticoids on blood pressure, consistent with other evidence that MR and GR in the brain mediate reciprocal neurochemical, neuroendocrine, and behavioral responses.

b. Hippocampal Degeneration. The hippocampus illustrates the importance of regional optimization of corticosteroid concentration. This organ contains the highest concentration of MR in the central nervous system (Reul and de Kloet, 1985; Krozowski and Funder, 1983; de Kloet *et al.*, 1984). It is extremely vulnerable to corticosteroid hormones. Chronic glucocorticoid exposure coupled with other chronic insults (e.g., aging, ischemia) results in the degeneration of specific hippocampal (CA1, CA3) fields (Sapolsky and Pulsinelli, 1985; Dokas, 1990). Glucocorticoid absence also results in specific hippocampal degeneration (Sloviter *et al.*, 1989). There must therefore be a mechanism to maintain glucocorticoids (e.g., corticosterone) in specific hippocampal fields within a defined range of intracellular concentration. Possibly, the survival of these cells requires a persistent occupation of the MR or GR by steroid ligand at some tonic level (Sloviter *et al.*, 1989). That the effects are direct is supported by the observation that cells in culture as well as in intact brain are sensitized by excess glucocorticoid (Masters *et al.*, 1989; Freshney *et al.*, 1980).

c. Selectivity of Brain Corticosteroid Receptors: Proposed Role of 11-HSD. The source of the regional selectivity of brain receptors for mineralo- or glucocorticoid has not yet been determined. The evolution of hypotheses designed to investigate this question has paralleled those proposed for the kidney. For several years, the only serious contender for a selection mechanism was CBG, but this was withdrawn for the same reason as in the kidney (de Kloet and Reul, 1987; Funder, 1986); i.e., selectivity was not altered in animals with little or no circulating CBG. The suggestion that receptor selectivity was mediated by the local action of 11-HSD on glucocorticoids, similar to a parallel process in kidney, initiated a series of promising investigations. The starting point was the hypothesis that the excess corticosterone in blood entering cells represents an impediment to the ability of aldosterone to gain access to the MR in the absence of 11-HSD.

Oxidation of corticosterone to 11-dehydrocorticosterone by 11β-dehydrogenase occurred in widely distributed regions of the brain. Activities were highest in the hippocampus and cortex (Lakshmi *et al.*, 1991; Moisin *et al.*, 1990a), an observation that was confirmed by immunohistochemical staining of brain regions with 11β-dehydrogenase antibody (Lakshmi *et al.*, 1991; R. Rousseau *et al.*, 1972) and by *in situ* hybridization (Moisin *et al.*, 1990a) using cDNA corresponding to rat liver 11β-dehydrogenase (Agarwal *et al.*, 1989).

There is as yet no direct experimental evidence to show that 11-HSD is the selection mechanism for brain receptor. Correlation of 11-HSD activity and intensity of immunoreactive labeling is consistent with a protective mechanism. Using neuronal and glial markers to measure the distribution of 11-HSD-like antigen, it was found that 11-HSD was distributed in the hippocampus in the CA1-4 regions and the dentate gyrus. The distribution in the hippocampus and cortex coincided with the distribution of MR. Neuronal 11-HSD was found throughout the cell body and its projections. Consistent with the hypothesis that 11-

HSD is the selection mechanism for MR, receptor and enzyme were located within the same cell (Sakai *et al.*, 1990). The distribution was heterogeneous, with some neurons that contained MR showing no detectable 11-HSD immunoreactivity. The distribution studies suggest that the selection mechanism of 11-HSD protection of MR is retained within the individual neuron. The observation that the enzyme is localized in the neuronal nuclei and the discovery that in all brain regions investigated 11-HSD is found in glial cells suggest that its functions are complex.

In the hippocampus, as in other brain regions, gluco- and mineralocorticoids must both be present in some crucial, though as yet unknown, relationship for optimal function to occur. A neuron containing both GR and MR must be able to manipulate both glucocorticoid and mineralocorticoid levels to permit functionally adequate binding to the available receptors. This may require that corticosterone concentrations be adjusted to permit its occupancy of MR and/or GR in a way that is in accord with the needs of the cell, or alternatively, to permit glucocorticoid metabolism to proceed extensively in order for the MR to bind aldosterone. How 11-HSD activity is controlled to permit optimal neuronal function is not known. Several mechanisms are possible: (*a*) controlled synthesis and inactivation of enzyme; (*b*) control of activity based on availability of cofactor; (c) reversibility of enzyme, permitting net oxidation or reduction of 11-oxygenated steroid to occur.

In some regions of the brain, 11-HSD may mediate GR-dependent events. Cerebellum contains no measurable MR, but does have well-defined GR; 11-HSD is expressed as high activity accompanied by high levels of 11-HSD mRNA (Moisin *et al.*, 1990a). If 11-HSD serves any receptor-related function in cerebellum, it must only influence GR-dependent events. It has been suggested that 11-HSD may control glucose metabolism in the brain via GR. Inhibition of 11-HSD by glycyrrhetinic acid increased steroid-dependent uptake of 2-[^{14}C] deoxyglucose in the arcuate nucleus, proptic area, cortex, hippocampus, and paraventricular nucleus (Seckl *et al.*, 1991). Glial cells contain GR (McGinnis and de Vellis, 1981), but no MR, respond to glucocorticoids, and contain 11-HSD. There are thus several examples of cell types in which the resident 11-HSD may serve cell-specific functions depending on their receptor content.

E. Leydig Cells, Stress, and 11-HSD

An extensive literature has accumulated that shows that the testis synthesizes less testosterone when exposed to pharmacological levels of circulating corticosteroid, and that the diminished responses are receptor mediated (Phillips *et al.*, 1989). Extending the idea first proposed for kidney function, it was suggested that 11-HSD protects the production of testosterone by Leydig cells against the inhibitory effects of glucocorticoids. That testicular 11-HSD is restricted to the Leydig cells is consistent with this hypothesis.

By inactivating cortisol (corticosterone in the rat) 11-HSD acts as an enzymatic barrier. This testicular barrier is overwhelmed when the level of circulating glucocorticoid exceeds a threshold defined by the enzyme's ability to oxidize the steroid. It was recently found that 11-HSD is absent from rat Leydig cells prior to the twenty-fifth postnatal day (Phillips *et al.*, 1989; Haider *et al.*, 1990). This observation suggested that prior to 25 days of age, corticosterone cannot be inactivated and thus contributes to the prepubertal suppression of testosterone production. Subsequently, as enzyme is expressed and corticosteroid is oxidized, inhibition of testosterone is overcome. As the animal ages, it is only possible to inhibit testosterone production with amounts of cortisol and corticosterone that exceed the oxidative capacity of 11-HSD, or by glucocorticoid analogs, such as dexamethasone, that are poor substrates for the enzyme. The 11-HSD inhibitor carbenoxolone increases the testosterone suppressive effects of corticosterone, an effect in accord with predictions (Abayasekara *et al.*, 1990). The synthetic glucocorticoid dexamethasone inhibits testosterone secretion by Leydig cells, but since, unlike corticosterone, it is not a substrate of 11-HSD, its effect is not increased by 11-HSD inhibitors (Monder *et al.*, 1992). The mineralocorticoid aldosterone has no effect on testosterone production (Abayasekara *et al.*, 1990; Monder *et al.*, 1992), since Leydig cells have no MR (R. R. Sakai, M. Hardy, and C. Monder, unpublished observations).

F. Mammary Gland

In the mammary gland, glucocorticoids are required for the synthesis of casein, lactalbumin, and other proteins, through a GR-dependent process (Jahn *et al.*, 1987). Quirk *et al.* (1990a) have found 11-HSD in the epithelial and adipose tissue of pregnant and lactating mammary gland of rats. The enzyme is 20-fold higher in adipocytes than epithelial cells and diminishes in both cell types as pregnancy progresses to reach low levels in lactating glands. The authors propose that 11-HSD decreases local concentration of corticosterone by the formation of the inactive 11-dehydrosteroid metabolite, and thus prevents premature milk production (Quirk *et al.*, 1990a,b). The presence of MR in breast tissue (Quirk *et al.*, 1983) suggests that the role of 11-HSD in

the mammary gland may involve the participation of corticosteroids on salt and water metabolism (Molina *et al.*, 1990) as well as on milk protein production.

X. Epilogue

In this article, we have attempted to provide a historical perspective into the conceptual evolution of 11β-hydroxysteroid dehydrogenase from its pedestrian origin as an enzyme that catalyzes reversible inactivation of corticosteroids to its currently more prestigious role as mediator of steroid–receptor interactions. The recent surge of interest in 11-HSD was powered by two factors. The first was the recognition of clinical disorders whose symptomatology could be rationalized as being due to defects in 11-HSD expression. The second was the development of the tools—antibodies, cDNA—that facilitated exploration of the enzyme at the molecular level. With the use of these probes, investigators have developed hypotheses implicating the interplay of corticosteroids, 11-HSD, and steroid receptors in the etiology of juvenile hypertension, and in normal renal function. The success of this endeavor has inspired further exploration of the possibility that the principles that emerged from the study of the kidney also apply to other organs. The current state of these investigations has been summarized in this article. The significance of 11-HSD as a mediator of steroid–receptor interaction cannot, however, be unique. The concepts emerging from studies with this corticosteroid-metabolizing enzyme ought to apply as well to enzymes participating in the metabolism of other classes of steroid. Thus, there is reason to believe that the answers emerging from the questions posed in this article may find application elsewhere in steroid biology.

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Central hypertensinogenic effects of glycyrrhizic acid and carbenoxolone

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Gomez-Sanchez, Elise P., and Celso E. Gomez-Sanchez. Central hypertensinogenic effects of glycyrrhizic acid and carbenoxolone. *Am. J. Physiol.* 263 (*Endocrinol. Metab.* 26): E1125-E1130, 1992.—The apparent mineralocorticoid excess syndrome of patients ingesting large amounts of licorice or its derivatives is thought to be caused by the antagonism by these compounds of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). 11β-HSD inactivates cortisol and corticosterone, allowing the more abundantly produced glucocorticoids access to the mineralocorticoid receptor (MR) in the kidney, where they act as mineralocorticoids. We have found that the infusion of both glycyrrhizic acid, an active principle of licorice, and carbenoxolone, a synthetic analogue, into a lateral ventricle of the brain [intracerebroventricular (icv)] of a rat, at a dose less than that which has an effect when infused subcutaneously, produces hypertension. Furthermore, the hypertension produced by the oral administration of carbenoxolone or glycyrrhizic acid is blocked by the icv administration of RU 28318, an MR antagonist, at a dose below that which has an effect on blood pressure when infused subcutaneously. While the oral administration caused saline polydipsia and polyuria typical of chronic systemic mineralocorticoid excess, the icv licorice derivatives produced hypertension without affecting saline appetite. Sensitizing the rats to mineralocorticoid hypertension by renal mass reduction and increasing salt consumption was not necessary for the production of hypertension. These findings provide additional evidence for a central role in blood pressure control by mineralocorticoids that is distinct from their renal effects. They also suggest that more is involved in licorice-induced hypertension than only inhibition of 11β-HSD.

hypertension; licorice; mineralcorticoids; RU 28318; steroid 11β-hydroxysteroid dehydrogenase

ALDOSTERONE acts through type I receptors, or mineralocorticoid receptors (MR), in the kidney to produce sodium retention and potassium and hydrogen ion excretion. The MR is widely distributed and is present in the colon, parotid, vasculature, and, in particular, specific areas of the brain (5, 13). The affinity of isolated MR from various sources, including expressed MR cDNA in COS cells, is similar for aldosterone, corticosterone, and cortisol (3, 4, 16). MR, regardless of the source, are physicochemically identical (16, 32), and appear to be a product of the same cDNA (3). Corticosterone and cortisol normally do not act as mineralocorticoids in the kidney in vivo. Specificity, originally thought to be intrinsic to the receptor, has been shown to be conferred extrinsically by corticosterone/cortisol-binding globulin (CBG), which reduces free circulating glucocorticoid available to the receptor, and by 11β-hydroxysteroid dehydrogenase (11β-HSD). 11β-HSD reversibly converts corticosterone and cortisol to the inactive 11-dehydrocorticosterone and cortisone (7, 9, 12). The location of the 11β-HSD enzyme has been controversial. It appears that 11β-HSD is expressed in some mineralocorticoid target cells along with the MR, thus serving as an autocrine control, as well as in cells proximate to MR-containing cells, serving a paracrine function (6, 9, 21, 24).

Under normal conditions, most MR in the rat brain are almost fully occupied by corticosterone, while occupation of the type II receptor, or glucocorticoid receptor (GR), for which corticosterone has less affinity, is less complete and follows the circadian rhythm of glucocorticoid levels (7). It has been suggested that the occupation of the MR in the brain, particularly in the hippocampus, by corticosterone at low, physiological serum levels is possible because CBG does not penetrate the blood-brain barrier (7, 9) and because the activity of 11β-HSD in this organ is negligible (9, 12). However, in situ hybridization techniques have demonstrated the presence of 11β-HSD in the brain (19), as well as the kidney. Whether 11β-HSD is bioactive in any, all, or only specific parts of the brain is controversial (9, 19, 21). There are different tissue-specific forms and regional activity of the 11β-HSD enzyme (20) that may account for the apparent "glucocorticoid-selective" MR in some parts, particularly the hippocampus, of the brain, in contrast to the "aldosterone-preferring" MR in the anterior hypothalamus (7, 18). Seckl et al. (27) have reported that 11β-HSD inhibition by glycyrrhetinic acid in vivo in rats increased 2-deoxy-[^{14}C]glucose use in those areas of the brain where 11β-HSD mRNA expression has been documented. Corticosterone and aldosterone have different actions in some areas of the brain, even though both are thought to be acting with the same affinity and through the same receptor. Aldosterone antagonizes important central nervous system (CNS) effects of corticosterone (7, 26); corticosterone blocks the hypertension induced by the intracerebroventricular (icv) infusion of aldosterone (13, 15).

Apparent mineralocorticoid excess is a rare hypertensive syndrome in which patients have all of the manifestations of excessive production of mineralocorticoids, including hypokalemia, but steroid measurements are normal or low. The defect has been identified as a deficiency in 11β-HSD (11, 28, 30, 31). The pseudohyperaldosteronism, including hypokalemia and low-renin hypertension, produced by excessive licorice consumption and the treatment of peptic ulcers with licorice derivatives or their synthetic analogues has been attributed to the inhibition of this enzyme, allowing the more abundant circulating cortisol/corticosterone access to the MR in the kidney (9). Licorice derivatives and the synthetic analogue carbenoxolone have been used to study the mechanisms responsible for the syndrome of apparent mineralocorticoid excess, as well as the extrinsic factors conferring apparent ligand specificity to the MR (8, 10, 22). We herein describe studies of the central and

systemic effects of the icv, subcutaneous (sc), and oral administration of glycyrrhizic acid, a derivative of licorice, and carbenoxolone, a synthetic analogue, on the blood pressure using the specific MR antagonist RU 28318 (14) to inhibit the MR.

METHODS

Cannulas were placed into the right lateral cerebral ventricles of male outbred Sprague-Dawley rats weighing 180–200 g, using aseptic surgical technique under a combination of fentanyl and droperidol (Innovar-Vet, Pitman-Moore), 0.01 ml/100 g body wt sc, as preanesthetic and isoflurane as anesthetic. Rats received standard food (0.3% NaCl) and tap water or 0.9% saline ad libitum to amplify the hypertension as detailed below. Implanted miniosmotic pumps (Alzet 2002, Alza, Palo Alto, CA), which delivered 0.49 ± 0.02 μl/h for 14 days, were used for icv and sc infusions. Pumps were changed on *day 14* under isoflurane anesthesia, and pumps of the same lot were used throughout the experiment to ensure consistency. Carbenoxolone, RU 28318, and corticosterone were dissolved in cerebrospinal fluid (CSF) or 0.86% NaCl with 10% propyleneglycol for icv and sc infusion. A potassium gluconate solution that delivered the same amount of K^+ as the RU 28318 solution was used as control for the mineralocorticoid antagonist experiments (14). Reagents were purchased from Sigma, except for the RU 28318, which was a gift from Roussell (Romaineville, France). All solutions were made and sterilized by filtration through 0.2-μm filters (Acrodisc 13, Gelman Scientific) immediately before filling and implanting the pumps. Oral carbenoxolone or glycyrrhizic acid was administered individually twice a day as 0.1 or 0.2 ml of a slurry mixed in corn syrup that the rats accepted readily. Indirect systolic blood pressures (IITC, Woodhills, CA) and weights were measured twice a week starting before treatment as described previously (13). Twenty-four- or forty-eight-hour urine volumes were measured once a week in a stainless steel rat metabolism cage.

Effect of icv administration of carbenoxolone: dose response. Carbenoxolone was infused icv at a rate of 0.3, 1.0, and 3.0 μg/h and sc at a rate of 3.0 μg/h into intact rats provided with 0.9% saline to drink ad libitum.

Effect of icv administration of carbenoxolone and corticosterone. Carbenoxolone was infused icv at a rate of 5.0 μg/h and corticosterone at a rate of 20 ng/h, alone and together. Two types of experiments were done. For one, the rats were intact and drank tap water ad libitum. For the other, the right kidneys were removed and the rats drank 0.9% saline ad libitum to be comparable to the classical maneuvers used to amplify mineralocorticoid hypertension.

Effect of oral administration of carbenoxolone with and without icv RU 28318. Carbenoxolone was administered orally in corn syrup 45 mg/kg twice daily for 10 days and increased to 90 mg/kg twice daily for the next 4 days to ascertain that the hypertensive effect was maximal; the control rats received corn syrup orally. RU 28318 was infused icv at 1.1 μg/h in one-half of the animals receiving carbenoxolone; the other animals received a potassium gluconate solution to supply the equivalent amount of K^+ icv. We have previously shown that 1.1 μg/h RU 28318 icv has no intrinsic effect on the blood pressure but protects the rat from the hypertension of systemic mineralocorticoid excess, while being well below the dose required to affect on the blood pressure when infused sc (13, 14). The rats were intact and drank tap water ad libitum.

Effect of oral administration of glycyrrhizic acid with and without RU 28318. The effects of both glycyrrhizic acid and carbenoxolone were studied because of evidence that carbenoxolone may have a larger range of effects, including the inhibition of 11-oxoreductase, than does glycyrrhizic acid (29).

Glycyrrhizic acid was administered orally in corn syrup 35 mg/kg twice daily for 14 days. RU 28318 was infused icv and sc at 1.1 μg/h in two of three glycyrrhizic acid groups; the other glycyrrhizic acid animals received a potassium gluconate solution icv to supply the equivalent amount of K^+ icv. Another group received corn syrup orally and the potassium gluconate solution icv. The rats were intact and drank tap water ad libitum.

Animals were killed at the end of the studies by CO_2 narcosis and asphyxiation. Autopsies, including dye infusions to check cannula placement, were done at the conclusion of the study, and data from any animal in which there was doubt about the delivery of the solutions or which had evidence of illness causing undue stress were eliminated from the experiment. At the time of the biweekly pump changes, if the catheter was found to be disconnected from the pump or cannula, the data from the preceding two weeks were discarded and the animal eliminated from the study. We started with 8–10 animals per group so that the groups were never reduced to fewer than 7 animals by the end of the experiment. Data were compared by analysis of variance and the Dunnett *t* and Fisher PLSD tests (StatView 512+, BrainPower, Calabazas, CA).

RESULTS

Carbenoxolone, 3 μg/h administered icv to intact rats drinking 0.9% saline ad libitum, increased the blood pressure of rats significantly ($P < 0.01$) within 3 days and was maximal by *day 5* (Fig. 1). There was no significant change in the blood pressure of rats receiving 0.3 μg/h CSF, or 1 μg/h carbenoxolone icv or 3 μg/h carbenoxolone sc over 14 days. No significant difference was found in rate of weight gain or 24-h urine volume between any groups in the icv studies. In separate studies it was found that doses of carbenoxolone >5 μg/h resulted in precipitation of the drug in the pump and cannulas.

The icv infusion of corticosterone at 20 ng/h, a dose known to inhibit the hypertension produced by the icv infusion of aldosterone (15) while having no effect in and of itself, did not significantly blunt the increase in blood pressure produced by icv carbenoxolone, nor did it have any effect on the blood pressure by itself (Fig. 2). There was no difference in urine volume or weight gain between groups in the same experiments. Removing one kidney and giving saline to drink did not alter the hypertension

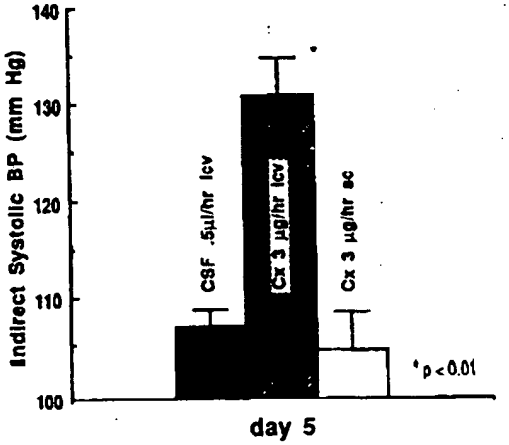

Fig. 1. Effect on indirect systolic blood pressure (BP) at *day 5* of intracerebroventricular (icv) and subcutaneous (sc) infusion of carbenoxolone (Cx) at 3.0 μg/h in intact rats drinking 0.9% saline ad libitum. CSF, cerebrospinal fluid.

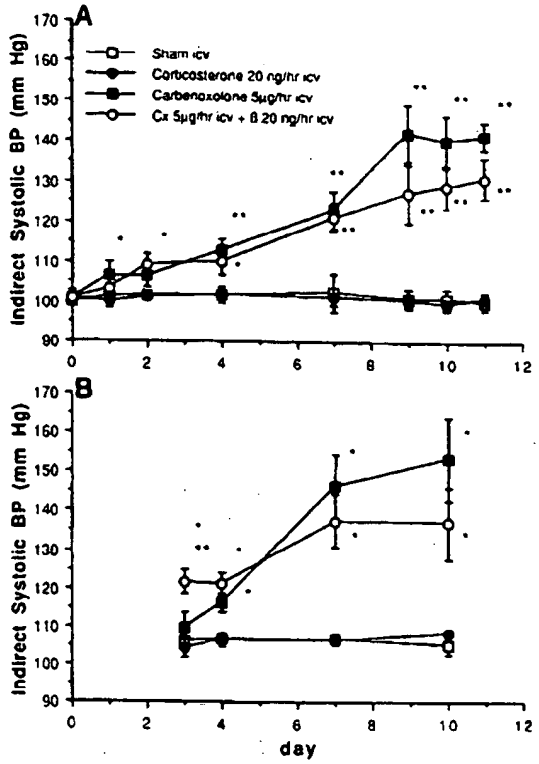

Fig. 2. Effect on indirect systolic blood pressure of icv infusion of carbenoxolone at 5.0 µg/h and corticosterone at 20 ng/h, alone and together, in nonsensitized rats (*A*; intact and drinking tap water ad libitum) compared with sensitized rats (*B*; one kidney removed and drinking 0.9% saline ad libitum). *β*, 11*β*-hydroxysteroid dehydrogenase. * $P < 0.05$. ** $P < 0.01$.

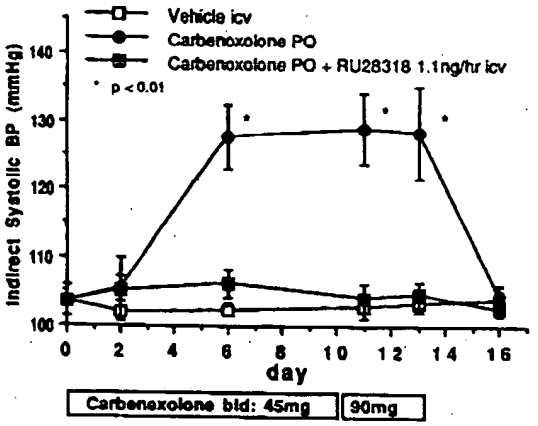

Fig. 3. Effect on indirect systolic blood pressure of oral administration of carbenoxolone in corn syrup at 45 mg/kg twice daily for 10 days, then 90 mg/kg twice daily for the next 4 days, while receiving an icv infusion of either RU 28318 at 1.1 ng/h or vehicle, in intact rats drinking tap water ad libitum.

produced by icv carbenoxolone or the effect of icv corticosterone. At *day 11* of the sensitization study there was a 41 and 39% difference in blood pressure between the controls and the icv carbenoxolone and icv carbenoxolone plus corticosterone, respectively, compared with 41 and 31% increases for the nonsensitized rats.

The blood pressure of intact rats drinking water and receiving oral carbenoxolone at 45 mg/kg twice daily increased significantly within 6 days from 105 mmHg to a plateau of 127 mmHg (Fig. 3). Doubling the dose to 90 mg/kg twice daily did not further increase the blood pressure. The icv infusion of 1.1 µg/h RU 28318 completely prevented the increase in blood pressure. We have shown in multiple studies, including those described below using glycyrrhizic acid instead of carbenoxolone, that the sc infusion of 1.1 µg/h RU 28318 is too low to affect the blood pressure. We have also reported that the icv infusion of the antagonist at three times this dose has no effect on the blood pressure of normal animals (14). The blood pressure in the animals receiving the icv control solution returned to normal within 3 days of discontinuing the oral administration of carbenoxolone. Orally administered carbenoxolone doubled the urine volume; this increase in urine volume was not prevented by the icv administration of the mineralocorticoid antagonist, which abolished the hypertension (Fig. 4). There was no difference in weight gain between groups.

The oral administration of glycyrrhizic acid at 35 mg/kg twice daily also significantly increased the blood pressure of intact rats drinking tap water. The icv infusion of 1.1 µg/h RU 28318 prevented the rise in blood pressure (Fig. 5). There was no difference in weight gain between groups.

DISCUSSION

The importance of the CNS in the development of mineralocorticoid hypertension has been well documented (5, 13). MR are found in the hippocampus, amygdala, lateral septum, and hypothalamus, particularly in the periventricular regions, areas known to be or suspected of being important in the regulation of adrenocorticotropic hormone (ACTH) release, arousal, fluid and fluid osmolality equilibrium, and the maintenance of normal blood pressure. The chronic icv infusion of aldosterone at a dose two orders of magnitude less than that necessary to produce hypertension when infused sc has been reported to produce hypertension in rats and dogs (21). The icv infusion of the mineralocorticoid antagonist RU 28318, at doses that have no effect on the blood pressure when given icv alone and that are ineffective as

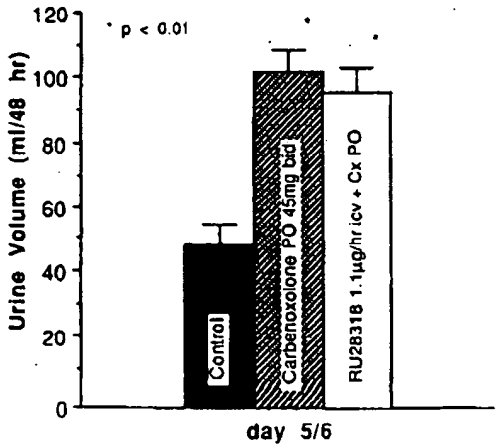

Fig. 4. Effect on 24-h urine volume of oral administration of carbenoxolone in corn syrup at 45 mg/kg twice daily for 10 days, then 90 mg/kg twice daily for the next 4 days, while receiving an icv infusion of either RU 28318 at 1.1 ng/h or vehicle, in intact rats drinking tap water ad libitum.

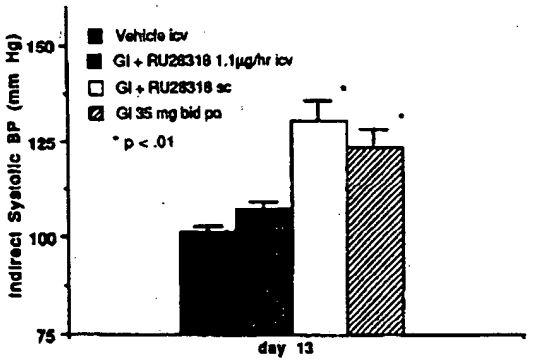

Fig. 5. Effect on indirect systolic blood pressure of oral administration of glycyrrhizic acid (GI) in corn syrup at 35 mg/kg twice daily, while receiving an icv infusion of either RU 28318 at 1.1 ng/h or vehicle, at *day 13* in intact rats drinking tap water ad libitum.

an antagonist when administered sc, blocks the hypertension of both the icv and systemic administration of aldosterone and the sc infusion of deoxycorticosterone acetate. The systemic, but not icv, aldosterone hypertension is associated with a chronically increased urine volume indicative of saline polydypsia/polyuria. The icv infusion of the antagonist prevents the rise in pressure produced by the systemic administration of aldosterone without preventing the associated polydypsia/polyuria (13, 14). These findings suggest distinct mineralocorticoid effects in the brain and kidney.

In the studies reported herein, the icv, but not sc, infusion of 3 μg/h carbenoxolone produced hypertension, implying that the site of action is in the brain. The hypertension produced by the oral and icv administration of carbenoxolone or glycyrrhizic acid resembles that of chronic systemic and icv aldosterone infusion in the amplitude of the increase in blood pressure and the effectiveness of mineralocorticoid receptor blockade by icv RU 28318 (13, 14). In addition, as with aldosterone, an increase in urine volume occurred only with the systemic, and not icv, administration of hypertensinogenic amounts of both licorice compounds. Blocking the hypertension of animals receiving oral carbenoxolone with the icv infusion of RU 28318 at doses too low to be effective when infused sc did not reduce their increase in urine volume. Classically mineralocorticoid-salt hypertension is associated with an initial retention of sodium and water followed by an "escape" from further retention and the establishment of a new equilibrium at a higher overall fluid volume. Polydypsia/polyuria may persist after reaching a balance with no additional net gain in water (13). Assuming that the carbenoxolone when given orally is causing a mineralocorticoid excess syndrome as far as the kidneys are concerned, one would expect initial sodium and water retention, followed by escape. The rats in these studies apparently were placed in metabolism cages after the water retention phase, assuming it occurred, after an equilibrium had been reached, because their urine output was consistently greater, not less, than that of controls. Weight gains were "real," not water gains, as evidenced by the fact that the weights of the oral carbenoxolone rats did not fall after the drug was withdrawn.

There was a consistent difference in the time of onset of the hypertension. Icv aldosterone hypertension takes from 7 to 11 days to become significant (13), while icv carbenoxolone hypertension was evident in 3–6 days. Considering the relatively long delay of onset, that of days rather than minutes or hours, it seems unlikely that this difference is due to a more rapid passage of the licorice compounds across the blood-brain barrier; it probably reflects a more basic difference in the mechanism of action. Removing one kidney and giving saline to drink did not exacerbate the hypertension produced by icv carbenoxolone. This was surprising because the classical way to amplify mineralocorticoid hypertension is to reduce renal mass and increase sodium consumption and because in the model of central mineralocorticoid hypertension, equihypertensinogenic doses of icv aldosterone in nonsensitized rats were nine times that of sensitized rats (13).

Glycyrrhizic acid and carbenoxolone are not thought to act as agonists at the receptor level because their affinity for the MR is negligible (2). They are presumed to work by inhibiting 11β-HSD, thereby removing the protection of the MR from corticosterone and allowing it to act as a mineralocorticoid (12). However, if 11β-HSD were active in the brain, and if it were inhibited by carbenoxolone, previous studies from our laboratory suggest that the resulting accumulation of corticosterone would not be expected to increase blood pressure. An additional difference between the icv aldosterone and icv carbenoxolone models is that the icv infusion of corticosterone, at a dose that would have been expected from our previous work to antagonize the icv aldosterone model, had no effect on the blood pressure of rats receiving icv carbenoxolone. It is assumed that the inhibitory action of icv corticosterone on icv aldosterone hypertension is mediated by the MR because RU 26988, a selective GR agonist, had no effect when infused alone or in combination with aldosterone (15).

While most reported studies indicate that carbenoxolone does not affect the mineralocorticoid activity of aldosterone (25), others suggest that it enhances the sodium retention produced by aldosterone and 11-deoxycorticosterone (23). Glycyrrhetinic acid has been found to inhibit the hepatic 5β-reductase and 3β-HSD but not the 5α-reductase or 3α-HSD. Another proposed mechanism for the enhancement of mineralocorticoid activity by licorice derivatives is the accumulation of aldosterone, deoxycorticosterone, and 11-deoxycorticosterone and their biologically active 5α-dehydro derivatives due to the inhibition of the 5β-reductase and 3β-HSD enzymes, as well as of glucocorticoids due to 11β-HSD inhibition (17).

Patients with apparent mineralocorticoid excess appear to be deficient in 11β-dehydrogenase but not 11-oxoreductase enzyme activity (30). While it has been assumed that 11β-HSD is an enzyme complex consisting of an 11β-dehydrogenase and a distinct 11-oxoreductase (9, 22, 30), a rat cDNA has been cloned and expressed as a single enzyme that interconverts cortisol/corticosterone to cortisone/11-dehydrocorticosterone (1). It has been reported that glycyrrhizic acid and carbenoxolone are not identical in their clinical activities and that glycyrrhizic acid inhibits the conversion of cortisol/corticosterone

to cortisone/11-dehydrocorticosterone unidirectionally, while carbenoxolone inhibits both the dehydrogenase and reductase directions (29). In our studies, the activity of glycyrrhizic acid and carbenoxolone were similar.

There is evidence for yet another mechanism of action of carbenoxolone. The MR is either missing or defective in patients with pseudohypoaldosteronism. Funder (10) has reported that the administration of carbenoxolone with a selective GR agonist in patients with pseudohypoaldosteronism and in adrenalectomized rats alters the function of the glucocorticoid, causing it to produce the same renal effects, Na^+ retention and K^+ excretion, as a mineralocorticoid would, presumably by causing GR-ligand complexes to act as activated MR. The animals in our experiments had intact adrenals; in fact, the mineralocorticoid effects of licorice depend on intact adrenal glands or replacement corticosteroids (9). Normally, most of the MR and many of the GR of the brain, depending on the area, are tonically bound by corticosterone, even in the unstressed rat (7, 26). While the concomitant icv infusion of corticosterone blocks icv aldosterone hypertesion, the icv infusion of a selective glucocorticoid, presumably to the GR only, does not antagonize icv aldosterone hypertension. If there are two classes of MR in the brain, as has been postulated by De Kloet (7), carbenoxolone and glycyrrhizic acid may be altering the "corticosterone-preferring" MR to functionally "aldosterone-preferring" MR. If carbenoxolone were producing hypertension by "recruiting" GR and/or corticosterone-preferring MR bound to endogenous corticosterone to the pool of functionally activated MR, not only might the same cellular response be elicited as by activated MR in a mineralocorticoid-sensitive central blood pressure control area, but, more important, it might also remove the receptors that mediate the inhibition of icv aldosterone hypertension. This might explain why icv corticosterone, when given with carbenoxolone, neither increased the blood pressure, because the receptors were already surfeited, nor decreased it, because they were being diverted from their usual role of buffering the hypertensinogenic effect of aldosterone. The more rapid induction of hypertension by licorice compounds compared with aldosterone may be due more to the removal of local inhibitory effects than to the recruitment of more functional MR. The yin-yang relationship of the two classes of corticosteroids has been described elsewhere, including in the brain (7).

These data provide additional evidence for a central role in blood pressure control by mineralocorticoids that is distinct from their renal effects and that involves a complex homeostatic relationship between the two classes of corticosteroids in their central effects on blood pressure. They suggest that our understanding of functional specificity of the corticosteroid receptor-ligand complex, particularly in the brain, is incomplete. Finally, these studies indicate that more is involved in licorice-induced hypertension than the inhibition of 11β-HSD.

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Inhibition of 11β-Hydroxysteroid Dehydrogenase Obtained from Guinea Pig Kidney by Furosemide, Naringenin and Some Other Compounds

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Inhibition of 11β-hydroxysteroid dehydrogenase (11β-OHSD) can cause excess mineralocorticoid effects and hypokalemia. Several substances causing hypokalemia (glycyrrhizic acid in licorice and gossypol) inhibit this enzyme. We tested other compounds for activity to inhibit 11β-OHSD in guinea pig kidney cortex microsomes with NADP as cofactor and cortisol as substrate. Furosemide was an inhibitor while bumetanide was not, indicating a mechanism for the increased K^+ excretion caused by furosemide compared with bumetanide. Naringenin (found in grapefruit juice), ethacrynic acid, and chenodeoxycholic acid had inhibitor IC_{50} values similar to glycyrrhizic acid. We conclude that various compounds can inhibit this enzyme and may play a role in K^+ metabolism and adrenocorticosteroid action.

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INTRODUCTION

The syndrome of apparent mineralocorticoid excess, first described by Ulick, Ramirez and New in 1977 [1], has led to much research on the enzyme 11β-hydroxysteroid dehydrogenase (11β-OHSD). Deficient activity of this enzyme in children leads to their inability to oxidize cortisol to inactive cortisone, providing high cortisol levels in the kidney which activate renal mineralocorticoid receptors and cause hypertension and hypokalemia. Subsequently, the mechanism of licorice-induced hypermineralocorticoidism was shown to be the inhibition of 11β-OHSD by the active principle of licorice, glycyrrhizic acid. Since then, much research has been done to explore the role that this enzyme plays in regulating the interactions of cortisol with mineralocorticoid and glucocorticoid receptors [2–6].

Gossypol, a polyphenolic constituent of cotton seed, has been studied in China as a potential oral contraceptive for men because it suppresses sperm motility and formation without affecting testosterone levels [7]. Some Chinese men who received gossypol developed hypokalemia although the cause remained obscure [7]. This is particularly remarkable since idiopathic hypokalemia, often associated with hyperthyroidism, occurs widely in China; in addition, normal Chinese men have serum potassium levels lower than men in four other countries, with 9% having values below 3.5 mmol/l [8].

In studies investigating how gossypol causes hypokalemia, we found that gossypol inhibited 11β-OHSD activity in guinea pig [9] and human [10] renal cortical microsomes. We also found that certain bioflavonoids inhibit rat liver 11β-OHSD [10]. Others have reported inhibition of the rat kidney enzyme by bile acids [11] and by steroidal and triterpenoid compounds [12], and inhibition of the rat liver enzyme by some substances in human urine [13]. We therefore decided to test a variety of compounds for their possible enzyme inhibiting effect, choosing drugs that can cause hypokalemia or sodium retention as a side effect, flavonoids from grapefruit juice that inhibit the oxidation of dehydropyridine calcium channel blocking drugs [15–17] or sterols in vegetable oils at concentrations of 100–500 mg/dl [14].

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MATERIALS AND METHODS

Chemicals and solutions

Sitosterol was a gift from Eli Lilly and Co. (Indianapolis, IN). Campesterol was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were purchased from Sigma Co. (St Louis, MO).

Most sterols, furosemide, ethacrynic acid, naringin and naringenin were dissolved in ethanol and diluted with methanol. Cholic acid, chenodeoxycholic acid, bumetanide, hydrochlorothiazide and spironolactone were dissolved in methanol. Phenylbutazone and indomethacin were dissolved in distilled water (pH 9). Glycyrrhizic acid was dissolved in distilled water. Corticosterone and hydrocortisone were dissolved in methanol (144 μmol/l) and kept at -4°C.

Enzyme preparation and measurement of 11β-OHSD activity

Kidney cortex was obtained from long-haired male Hartley guinea pigs. Tissue was homogenized in Krebs–Henseleit buffer as described previously [9], except for the use of a Tekmar Tissuemizer (Cincinnati, OH). Microsomes were prepared as described previously [9], except that they were diluted to a concentration of 1.25 mg protein/ml prior to storage at -70°C.

The enzyme activity in guinea pig kidney cortex microsomes was determined by measuring the rate of conversion of cortisol to cortisone. Five minutes before incubation, 2 μl of concentrated Triton DF-18 was added to each milliliter of the microsome suspension. The assay mixture contained 500 μl Krebs–Henseleit buffer (pH 7.2), 50 μl 5 mmol/l NADP, 40 μl of 144 μmol/l phosphate–sucrose buffer, 20–50 μl (25–63.5 μg) of microsome suspension in 0.01 M phosphate-sucrose buffer and various concentrations of each compound studied. This mixture was incubated in duplicate or triplicate. The total volume was 700 μl. Methanol concentration was kept at $< 10\%$. Control studies showed that this concentration did not inhibit the reaction. After 1 h of incubation at 37°C, the reaction was terminated by the addition of 3 ml methylene chloride and 20 μl 144 μmol/l corticosterone solution as the internal standard for assay of cortisone and cortisol.

The enzyme inhibition constant for furosemide was determined by adding furosemide in various amounts to achieve concentrations from 3.9 to 62 μmol/l in the incubation mixture and cortisol concentrations of 4, 8, and 16 μmol/l. The constants were obtained from a Dixon plot and a kinetic program (Chou J, Chou T-C: Michaelis-Menton analysis with microcomputers, Disk No. 1, Elsevier-Biosoft, 1989, Cambridge, England.

A modification of the HPLC method of Sang [9] was used to measure cortisol, cortisone and corticosterone in the microsomal incubation mixture. The steroids were extracted into methylene chloride by vortexing for 1 min, then centrifuged at 750 *g* for 15 min. The aqueous layer was removed by aspiration. 300 μl of 0.1 NaOH was added to the organic phase followed by vortexing for 30 s. The mixture was centrifuged and the aqueous layer removed. The organic phase was washed with 500 μl of milli-Q water (Millipore Corp., Bedford, MA). The 1.5 ml organic phase was transferred to clean glass tubes and dried by evaporation in a 45–50°C water bath. The residue was dissolved into 200 μl of methanol and 5 μl of this solution was injected into the HPLC apparatus. A standard curve for cortisol and cortisone was determined in duplicate in each enzyme experiment by using the same amount of microsome suspension after boiling to inactivate the enzyme. Standard curves were plotted as the ratio of peak height of cortisone (or cortisol) divided by the peak height of the internal standard vs steroid concentration. All unknown concentrations of cortisol and cortisone were determined from the standard curves from each experiment. The drug concentrations that inhibited the enzyme by 50% (IC_{50}) were estimated from at least 3 different concentrations of each compound evaluated by a dose–response program (Chou and Chou: Dose-effect analysis with microcomputers, Disk No. 2, Elsevier-Biosoft. 1989, Cambridge, England).

The HPLC apparatus used for quantitating the steroids consisted of a Waters Model 6000 A solvent delivery system, U6K injector, model 680 automated gradient controller, Waters 486 tunable absorbance detector and a BBC chart recorder (Model SE 120). The mobile phase contained methanol–water (30:70, v/v) at a flow rate of 1.0 ml/min. The Waters stainless steel Novapak C_{18} column (3.9 × 150 mm, 4μ) was kept at room temperature. The retention times for cortisone, cortisol and corticosterone were 6.5, 7.0 and 9.0 min, respectively.

RESULTS

The efficacy of the compounds tested to inhibit the NADP-utilizing form of 11β-OHSD from guinea pig renal cortex with cortisol as substrate is shown in Tables 1 and 2. Furosemide was the most potent inhibitor tested, with glycyrrhizic acid, naringenin, ethacrynic acid and chenodeoxycholic acid having potencies similar to each other but an order of magnitude less potent than furosemide. Data for glycyrrhizic acid, naringenin and naringin are shown in Fig. 1. The correlation coefficient (r value) for the computed values agreeing with the measured values for the potent inhibitors was 0.99 for furosemide, glycyrrhizic acid, and naringenin, 0.96 for ethacrynic acid and 0.86 for chenodeoxycholic acid. It was above 0.95 for all of the other compounds tested except for phenylbutazone which was 0.86.

The observations of enzyme inhibition by furosemide at varying concentrations of cortisol is

Table 1. Inhibition of 11β-OHSD by various compounds

Compound	IC_{50} (μmol/l)	Concentrations tested (μmol/l)
Furosemide	59	12, 50, 100, 200, 500, 1000
Glycyrrhizic acid	254	132, 246, 529
Naringenin	336	12, 25, 50, 100, 1000, 2000, 5000
Ethacrynic acid	452	50, 100,200, 400, 2000
Chenodeoxycholic acid	513	200, 400, 600, 800
Phenylbutazone	1358	167, 667, 1344
Sitosterol	1395	500, 1000, 1500
Stigmasterol	1968	500, 1000, 1500
Naringin	2373	582, 1163, 1744
Cholic acid	3529	1250, 2500, 3750, 5000

Campesterol inhibited 33% at the highest concentration tested of 1000 μmol/l. Since a second higher point could not be measured because of limited solubility of the compound, an IC_{50} was not calculated.

shown as a double reciprocal plot in Fig. 2. Most of the lines converge near the ordinate. A Dixon plot indicated that the inhibition by furosemide is competitive. The enzyme kinetic constants were: $K_m = 8$ μmol/l and $V_{max} = 30$ nmol/μg microsomal protein/h. The K_i for furosemide was 7.7 μmol/l nearly the same as the K_m for cortisol.

DISCUSSION

We have tested a number of compounds for their ability to inhibit the NADP-utilizing form of 11β-OHSD from guinea pig renal cortex with cortisol as substrate. We found that furosemide is a much more potent inhibitor than glycyrrhizic acid, and that naringenin, ethacrynic acid and chenodeoxycholic acid inhibit with a potency almost equal to that of glycyrrhizic acid.

The compounds selected for study were chosen for a variety of reasons: the diuretics because they cause potassium loss with spironolactone as a control since it does not; glycyrrhizic acid and the bile salts as reference compounds, since data about these compounds have been published and therefore they can be used in this study to evaluate relative potency of the other compounds studied; naringin and naringenin because they are active compounds in grapefruit juice that inhibit a particular pathway of drug oxidation (cytochrome P_{450} 3A4) and we were curious to see if they also inhibited this oxidation pathway (11β-OHSD); the sterols since they are present in vegetable oils and have a structure suggesting that they might inhibit 11β-OHSD; and the cyclooxygenase inhibitors because they inhibit prostaglandin formation and cause salt retention.

The K_m of our enzyme preparation for cortisol (8 μmol/l) is similar to that of rat for corticosterone (2 μmol/l) found by Monder *et al.* [18]. Working with purified enzyme from rat liver (gift from Dr C. Monder), we have found an IC_{50} of 12 nmol/l for glycyrrhetinic acid [10], similar to the dissociation constant of the enzyme–inhibitor complex of 8 nmol/l reported by Monder *et al.* [18]. In a previous study from our laboratory, glycyrrhizic acid had an IC_{50} of 1994 μmol/l for guinea pig renal cortex microsomes with corticosterone as the substrate without Triton in the incubation mixture [9] compared with 254 μmol/l in the present study using Triton and cortisol as the substrate. Buhler *et al.* [12] working with rat kidney microsomes and corticosterone at 0.1 μmol/l, found an IC_{50} of 4 μM; in our study of guinea pig microsomes with a substrate concentration of 23 μmol/l we found an IC_{50} of 254 μM. Perschel *et al.* [11] working with rat kidney microsomes found cholic acid to inhibit this

Table 2. Compounds that failed to inhibit 11β-OHSD

Compound	Maximum concentration tested (μmol/l)
Bumetanide	2000
Hydrochlorothiazide	8000
Indomethacin	1100
Spironolactone	2000

The maximum concentration tested was limited by the solubility of the compound.

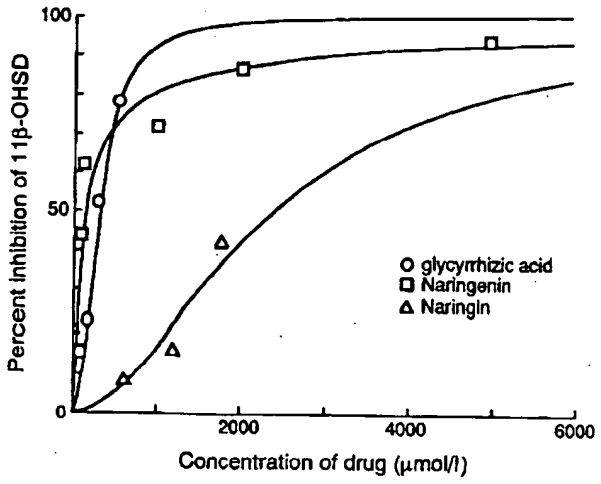

Fig. 1. Inhibition of 11[bt]β-OHSD by glycyrrhizic acid from licorice and flavonoids from grapefruit juice.

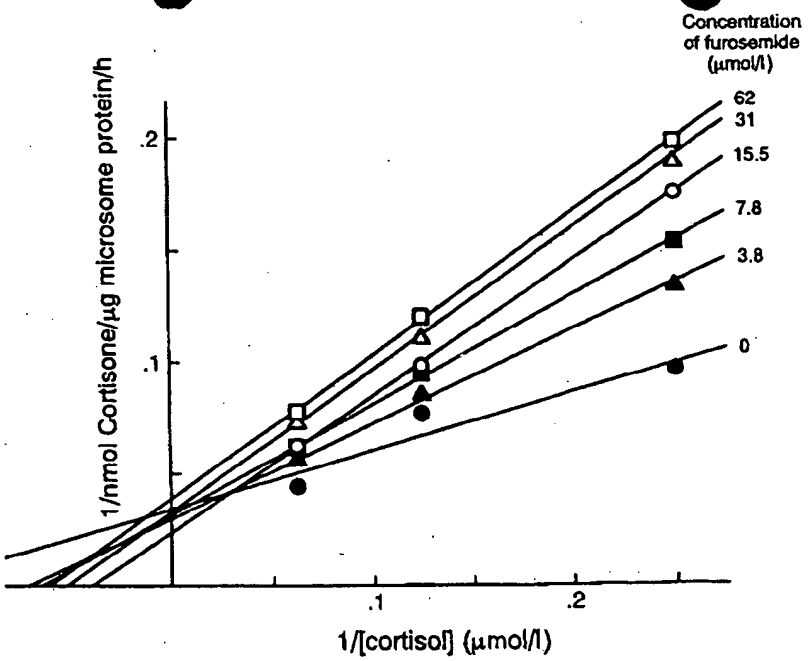

Fig. 2. Double reciprocal plot of 1/v 1/s for 11β-OHSD with varying concentrations of furosemide in incubation mixture. K_m for cortisol is 8 μmol/l. V_{max} is 30 nmol/μg microsomal protein/h. K_i for furosemide is 7.7 μmol/l.

enzyme at 1/27th the potency of chenodeoxycholic acid. We found it to be 1/7th the potency in our system.

We studied the NADP-requiring form of the enzyme that is present in most tissues rather than the NAD-requiring form that is present in the distal nephron [19–21]. Whether inhibition of the NAD-requiring enzyme is different from that of the NADP-requiring enzyme is not known. Since the mechanism of inhibition of glycyrrhetinic acid [18], gossypol [9] and furosemide (this study) is competitive, one might speculate that competitive inhibition of the NAD-requiring form of the enzyme by these compounds might also occur.

The fact that furosemide is an inhibitor of the enzyme while bumetanide is not may explain why furosemide causes more potassium excretion per unit sodium excretion than bumetanide [22–24]. It is excreted by patients with heart failure at a rate of 15–30 μg/min [25]. Assuming a 1 ml/min urine flow, the furosemide concentration would be 76 μM, compared with its IC_{50} of 59 μM in this study.

Three flavonoids: the sugar conjugates of naringenin, quercetin and kaempferol, along with some others are found in grapefruit juice [26]. These are hydrolyzed in the intestine to the aglycons which are absorbed. We found that naringenin inhibited the enzyme in this study, and previously that the flavonoids morin and quercetin were weak inhibitors [10]. The importance, if any, of these dietary constituents as *in vivo* inhibitors of this enzyme remains to be determined.

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PHARMACODYNAMICS AND DRUG ACTION

Grapefruit juice and its flavonoids inhibit 11β-hydroxysteroid dehydrogenase

Introduction: The enzyme 11β-hydroxysteroid dehydrogenase (11β-OHSD) oxidizes cortisol to inactive cortisone. Its congenital absence or inhibition by licorice increases cortisol levels at the mineralocorticoid receptor, causing mineralocorticoid effects. We tested the hypothesis that flavonoids found in grapefruit juice inhibit this enzyme in vitro and that grapefruit juice itself inhibits it in vivo.
Methods: Microsomes from guinea pig kidney cortex were incubated with cortisol and nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) and different flavonoids and the oxidation to cortisone measured with use of HPLC analysis. In addition, healthy human volunteers drank grapefruit juice, and the ratio of cortisone to cortisol in their urine was measured by HPLC and used as an index of endogenous enzyme activity.
Results: Both forms of 11β-OHSD requiring either NAD or NADP were inhibited in a concentration-dependent manner by the flavonoids in grapefruit juice. Normal men who drank grapefruit juice had a fall in their urinary cortisone/cortisol ratio, suggesting in vivo inhibition of the enzyme.
Conclusion: Dietary flavonoids can inhibit this enzyme and, at high doses, may cause an apparent mineralocorticoid effect. (CLIN PHARMACOL THER 1996;59:62-71.)

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The enzyme 11β-hydroxysteroid dehydrogenase (11β-OHSD) oxidizes cortisol to inactive cortisone. This enzyme in the kidney regulates the amount of mineralocorticoid activity there, because cortisol binds as avidly to the mineralocorticoid receptor as aldosterone does. Deficiency of this enzyme in children, first described by Ulick et al.[1] in 1977, causes high cortisol levels in the kidney that result in hypertension and hypokalemia. Licorice-induced hypermineralocorticoidism is probably due to the inhibition of 11β-OHSD by glycyrrhizic acid, the active principle of licorice.[2-4] Much research has been done since 1977 on syndromes of apparent mineralocorticoid excess.[5,6]

Gossypol, a polyphenolic constituent of cotton seed, has been studied in China as a potential male oral contraceptive, but hypokalemia developed in some Chinese men while they were taking it.[7] We found that gossypol inhibited 11β-OHSD activity in guinea pig[8] and human renal cortical microsomes.[9] Because there are structural similarities between gossypol and some flavonoids, we tested some of these and some other compounds, such as diuretics, that cause hypokalemia[9-10] and discovered that some inhibit this enzyme. Narin-

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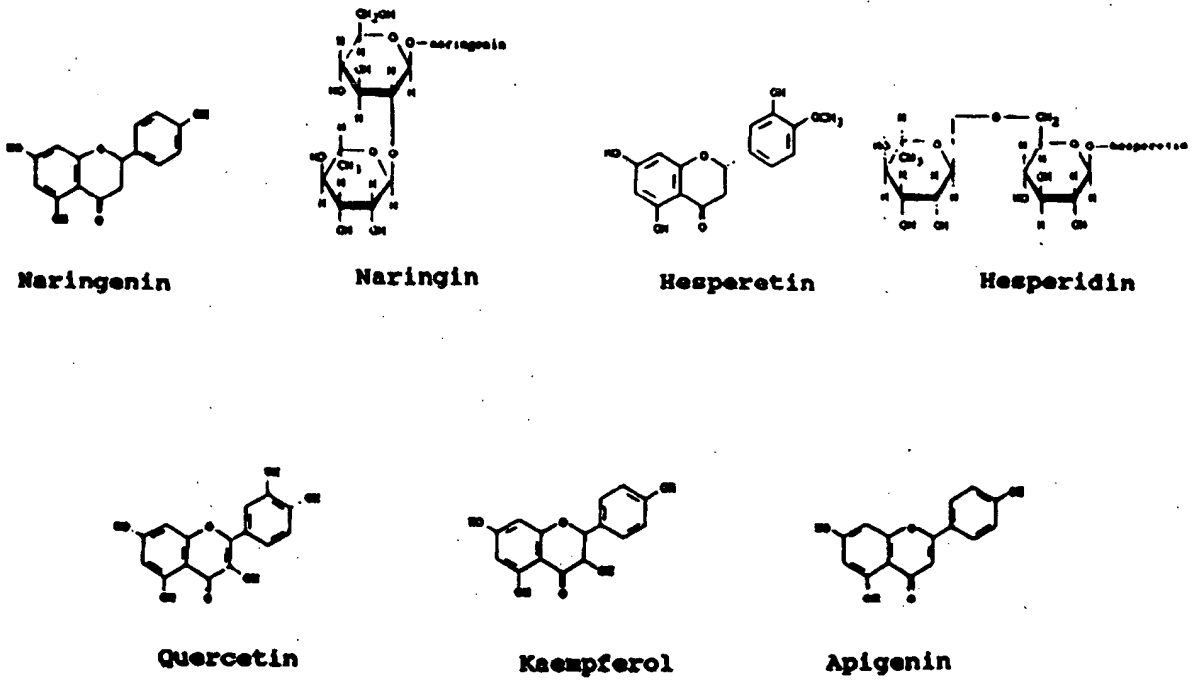


Structures of flavonoids.

genin, the aglycone of naringin, is a major flavonoid in grapefruit juice and inhibits this enzyme.[10] Recent work suggests that there are two isoforms of this enzyme, nicotinamide adenine dinucleotide (NAD)–dependent 11β-OHSD and nicotinamide adenine dinucleotide phosphate (NADP)–dependent 11β-OHSD with specific tissue distributions.[11-14] The effects of these flavonoids are worth study because about 25 mg of flavonoids has been recently estimated to be ingested daily in the diet,[15] whereas older studies cite as much as 1 gm per day.[16]

The objective of this study was to learn which other flavonoids in grapefruit juice inhibit 11β-OHSD in vitro and whether grapefruit juice inhibits the enzyme in vivo.

MATERIAL AND METHODS

In vitro study

Chemicals and solutions. All flavonoids (see Structures), cortisone, cortisol, corticosterone, NAD, NADP, 99.9% dimethyl sulfoxide (DMSO), and Sigma Diagnostic Total Protein Kit (cat. No. 690-A) were purchased from Sigma Chemical Co., St. Louis, Mo. All flavonoids were dissolved in DMSO. Cortisone, cortisol, and corticosterone were dissolved in methanol (J.T. Baker HPLC grade purchased from VWR Scientific, Piscataway, N.J.) (1.4 mmol/L) and kept at −4° C. NAD and NADP (5 mmol/L) were dissolved in Tris hydrochloric acid buffer (0.1 mol/L, pH 8.0).

Microsomal preparation and assay of 11β-OHSD activity. Guinea pig kidney cortex was obtained from long-haired male Hartley guinea pigs (Hilltop, Pa.). Tissue was homogenized by a Tekmar Tissuemizer (Cincinnati, Ohio). Microsomes were prepared, diluted to a concentration of 1.25 mg protein/ml as measured by the Sigma Diagnostics Total Protein Kit, and stored at −70° C. The enzyme activity in the microsomes was determined by measuring the rate of conversion of cortisol to cortisone in the presence of NAD or NADP as described previously.[8-10] Each flavonoid was studied with use of NAD and NADP as the cofactor. The conversion rates from cortisol to cortisone were determined, and the extent of inhibition was calculated. The drug concentrations that inhibited the enzyme by 50% (IC_{50}) were estimated from duplicate incubations at each concentration of at least three different concentrations of each flavonoid by use of the dose-response program of Chou and Chou (*Dose-effect Analysis with Microcomputers,* Elsevier-Biosoft, Cambridge, En-

gland, 1989). For each flavonoid studied, at least one concentration was above and one below the IC_{50}.

Analytical method for urinary cortisone and cortisol

We modified our HPLC method for measurement of these compounds from microsomal incubation mixtures.[9] The equipment consisted of a Waters Automated Gradient Controller with two Waters 6000A pumps (Waters Chromatography, Milford, Mass.). The injector was a Waters U6K and the detector was a Waters 486 Tunable Absorbance Detector set at a wavelength of 246 nm and 0.15 absorbance units full scale. The separation was performed with a Waters Nova-Pak C_{18} 3.9 × 150 mm stainless steel column (4 μm spherical particle size, pore size 60 Å, 7% carbon load, end-capped) or with a Waters μBondapak C_{18} 3.9 × 300 mm column (10 μm irregular particle size, pore size 125 Å, 10% carbon lead, end-capped). The peak areas were recorded on a SE120 plotter purchased through Waters Chromatography.

The mobile phase was methanol/water, initially at 70% water:30% methanol. Conditions were changed over the first 6 minutes to 56% water:44% methanol in a linear gradient that was then held isocratically for 14 minutes. The gradient was then reversed linearly to 70:30 over 3 minutes and the column equilibrated for 5 minutes before the next injection. The flow rate was 1 ml/min.

Procedure

To each 10 ml aliquot of every standard and sample (performed in duplicate) was added 40 μl of the 25 μg/ml corticosterone* as the internal standard. The samples were briefly vortexed to mix. One milliliter of 0.1 mol/L of sodium hydroxide was added to each test tube and again briefly vortexed to mix. Three milliliters of methylene chloride were added to each sample, capped with Teflon-lined screw tops, and rotated for 45 minutes on a mechanical rotator at approximately 20 rpm. The samples were centrifuged at 3000 rpm (1000*g*) for 15 minutes. The aqueous layer (top) was aspirated to waste. Again the samples were centrifuged for 10 minutes at 3000 rpm and the remainder of the aqueous phase was aspirated. A small spatula full of sodium chloride (~150 mg) was added to each sample, and any emulsion was broken up with a Pasteur pipet. The samples were then again centrifuged for 10 minutes. The organic layer was carefully transferred to clean test tubes and evaporated to dryness in a warm water bath (~45° C) under a stream of nitrogen. The residue was redissolved in 150 μl of HPLC grade methanol and injected into the HPLC.

The retention times were 16.5, 19.0, and 23.5 minutes for cortisone, cortisol, and corticosterone, respectively, on a Waters 10 micron, 300 × 3.9 mm stainless steel μBondapak C_{18} column. On a Waters 4 micron, 150 × 3.9 mm Nova-Pak, the retention times for cortisone, cortisol, and corticosterone were 12.8, 13.6, and 17.8 minutes. Levels measured in about 60 human urine samples ranged from 7.1 to 215.4 ng/ml for cortisone and 4.5 to 230.1 ng/ml for cortisol. The ratio of cortisone to cortisol was 0.2 to 5.7.

The absolute recovery was 70% for cortisol and 69% for cortisone. The interday coefficient of variation for cortisone was 6.5% for 25 ng/ml and 1.5% for the 100 ng/ml standard. For cortisol, the values were 6.3% for 25 ng/ml and 1.1% for 100 ng/ml. Cortisone dissolved in methanol was chromatographed and the peak was collected. The putative cortisone peak from extracted urine was also collected, and both fractions were scanned with a Varian Cary 219 spectrophotometer. The peaks had identical absorption spectra, with absorption maximums at 239 nm. (The *CRC Handbook of Chemistry and Physics* states that the absorption maximum of cortisone in alcohol is 237 nm).

All samples were assayed twice in duplicate. Standard curves for cortisone and cortisol were determined and plotted as in the in vitro study. Concentrations of these steroids in unknown samples were extrapolated from these standard curves.[10]

In vivo preliminary study

Six male volunteers aged from 35 to 65 years (two investigators and four other members of the Department of Pharmacology) who were living at home gave daily morning urine samples for 4 days. They then drank grapefruit juice, requested to be at a dose of a quart a day, for 7 days, and gave daily morning urine samples on the last 4 days of this period. After a 3-day washout period, the subjects again gave daily morning urine samples for 4 days.

*Corticosterone is excreted by humans at a rate that averages 6 μg/24 hours[17] or less than of 1% of 1.5 to 4.0 mg/24 hour production rate.[18] Thus the concentration from endogenous sources is less than 10% of that added, a negligible amount for this assay.

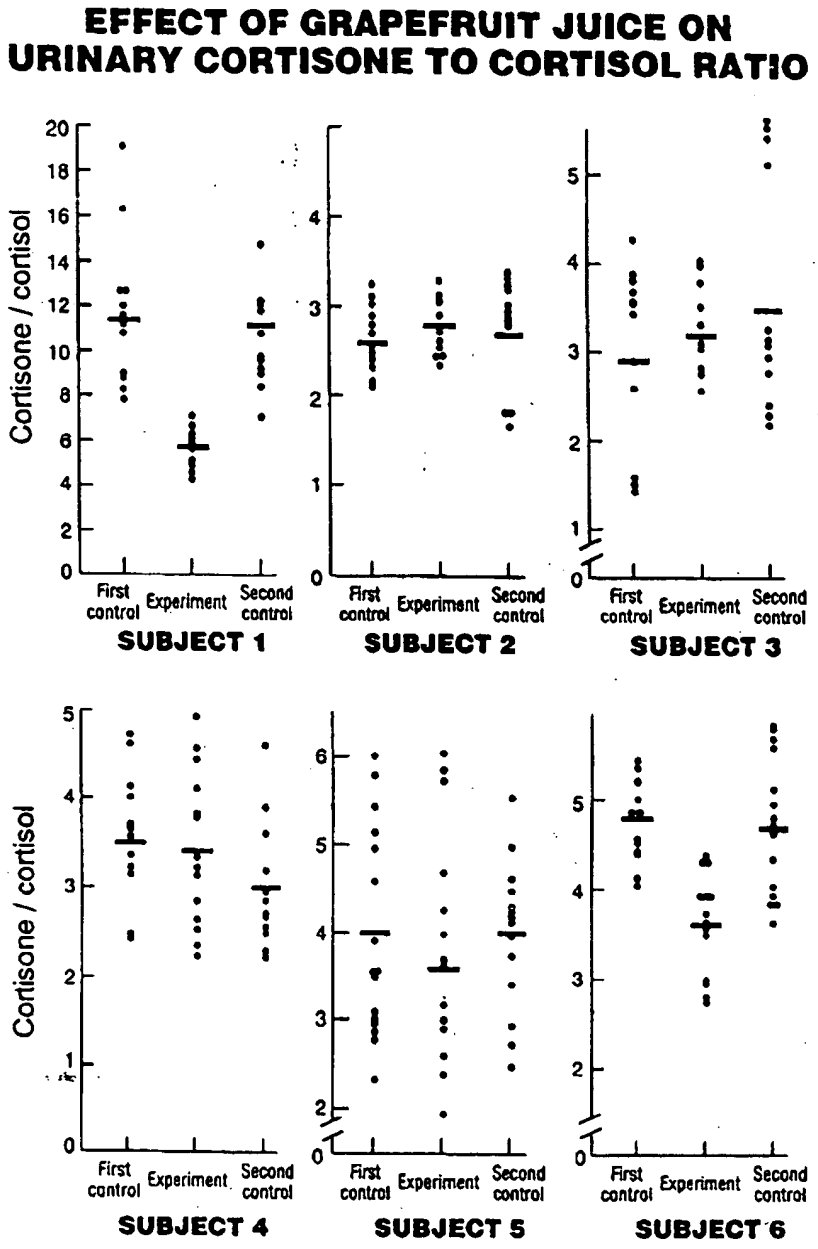


Fig. 1. Preliminary study results in six subjects living at home. Subjects 1 and 6 were two of the authors, who are known to have consumed the full amount of grapefruit juice.

The cortisone and cortisol concentrations were measured in each urine sample. The two investigators (subjects 1 and 6) had a decrease in the ratio of urinary cortisone to cortisol during the grapefruit juice period compared with the control periods before and after grapefruit juice (mean ± SD for subject 1 was 11.4 ± 3.1, 5.7 ± 0.9, and 10.2 ± 2.1; mean ± SD for subject 6 was 4.8 ± 0.4, 3.6 ± 0.6, and 4.7 ± 0.8). The other four subjects had no significant change. All data are shown in Fig. 1. Subjects 1 and 6 then volunteered for the dose-response study.

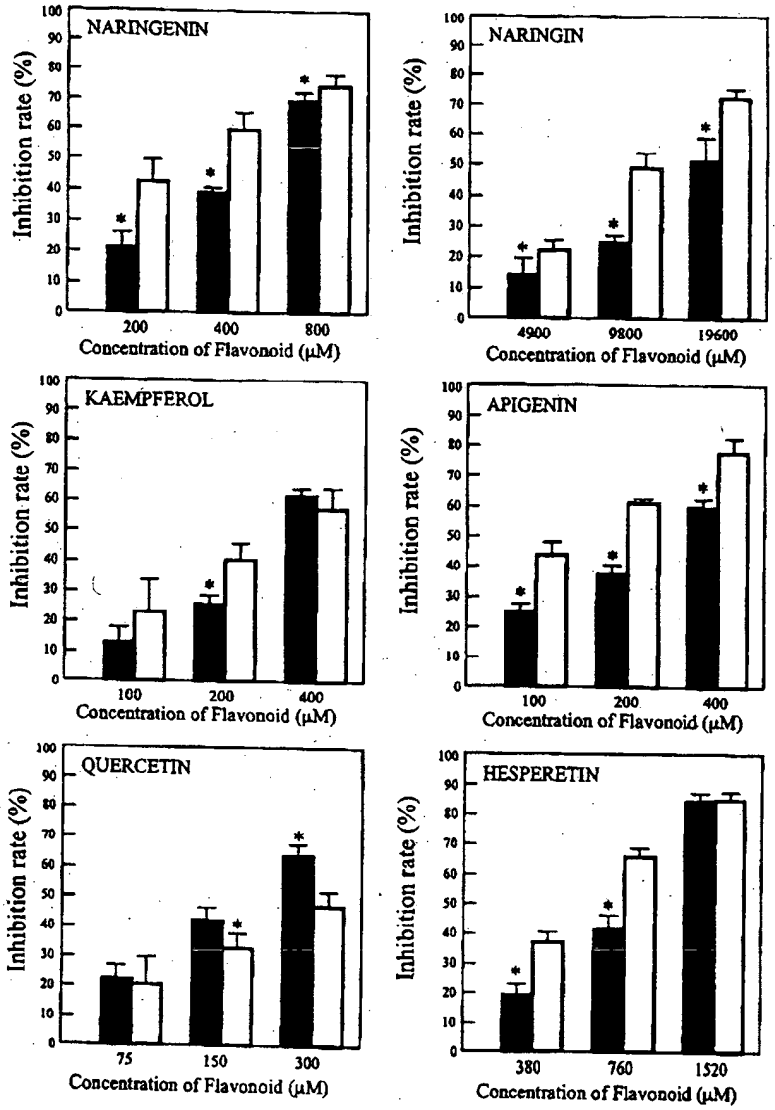


Fig. 2. Concentration-response relationships for the inhibition of 11β-hydroxysteroid dehydrogenase by different flavonoids with use of nicotinamide adenine dinucleotide (NAD; *solid bars*) or nicotinamide adenine dinucleotide phosphate (NADP; *open bars*) as a cofactor. $^*p < 0.05$.

In vivo dose-response study

These two volunteers (subjects 1 and 6) gave urine samples for the last 4 days of four 7-day study periods. (1) First control period: Each subject collected a 10-hour (7 AM to 5 PM) urine sample daily for 4 days (one subject missed 1 day of sample collection). (2) Low-dose period: Each subject drank 950 to 1060 ml grapefruit juice a day for 7 days and gave 10-hour urine samples for the last 4 days of the 7-day period. (3) High-dose period: Each subject drank 1900 to 2100 ml (double volume of low-dose period) grapefruit juice for 7 days and gave daily 10-hour urine samples for the last 4 days. (4) Second control period: Each subject gave daily 10-hour urine samples for 4 days after 3 days of a washout period.

In vivo metabolic balance study

Protocol. Two different healthy male volunteers (aged 26 and 31 years), not previously screened for responsiveness to grapefruit juice, gave informed written consent and were admitted to the clinical research center for 3 weeks. An evaluation before the study

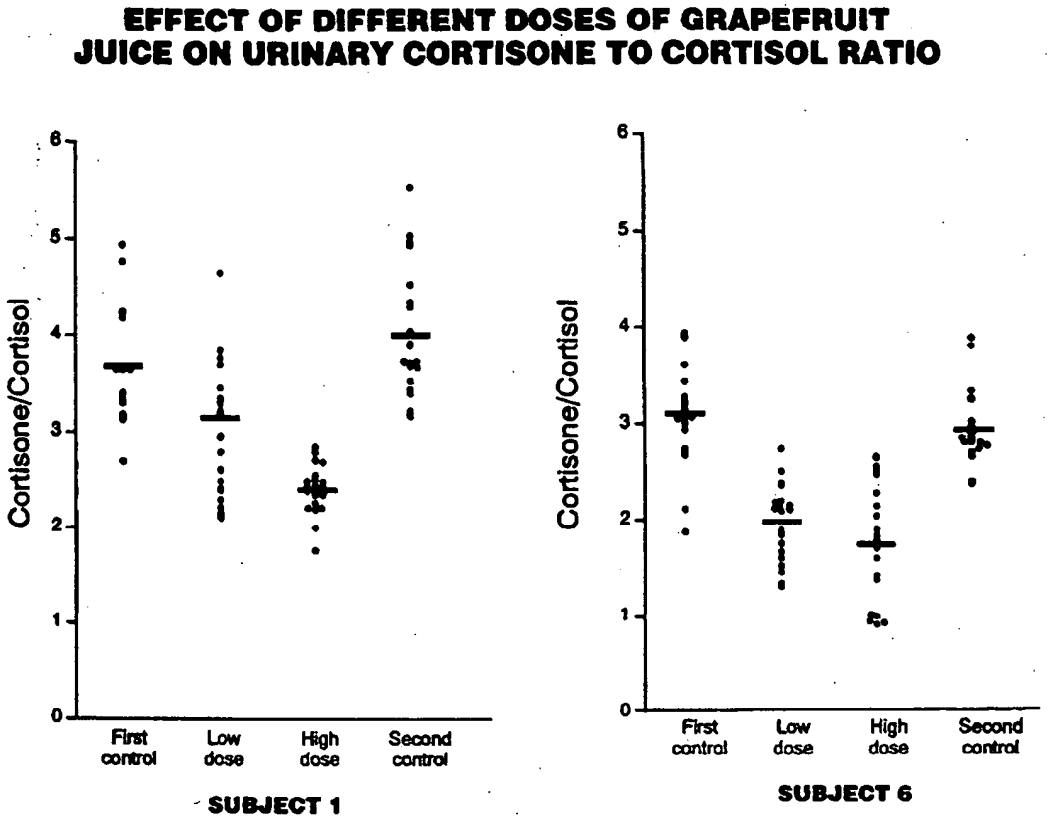


Fig. 3. Urinary cortisone/cortisol ratios in subjects in dose-response study. Each period include four daily urine collections. Each urine sample was assayed twice, each assay in duplicate. Each *point* represents a single assay (four points per daily urine).

showed normal physical findings, serum chemistry, hematology, and ECG for both subjects. They ate a diet that had a constant amount of sodium and potassium during the study (potatoes, bananas, and lemonade during control periods to balance the grapefruit juice during the experimental period). Their blood pressures and body weights were measured daily. Twenty-four-hour urine was collected for free cortisone, cortisol, Na^+, and K^+ for the last 4 days of three 7-day study periods. Blood samples were drawn for Na^+ and K^+ for the same periods. Plasma renin activity, aldosterone and cortisol, and urinary aldosterone excretion were measured at the end of each period. The first and third weeks were the control periods. The second week was the experimental period in which 1500 ml grapefruit juice (100% from concentrate, Ocean Spray Cranberries Inc., Lakeville, Mass.) was consumed daily.

Statistics. The Bonferroni *t* test after a one-way ANOVA was used to assess statistically significant differences. Statistical significance was assumed when the corresponding *p* values were lower than $\alpha = 0.05$.

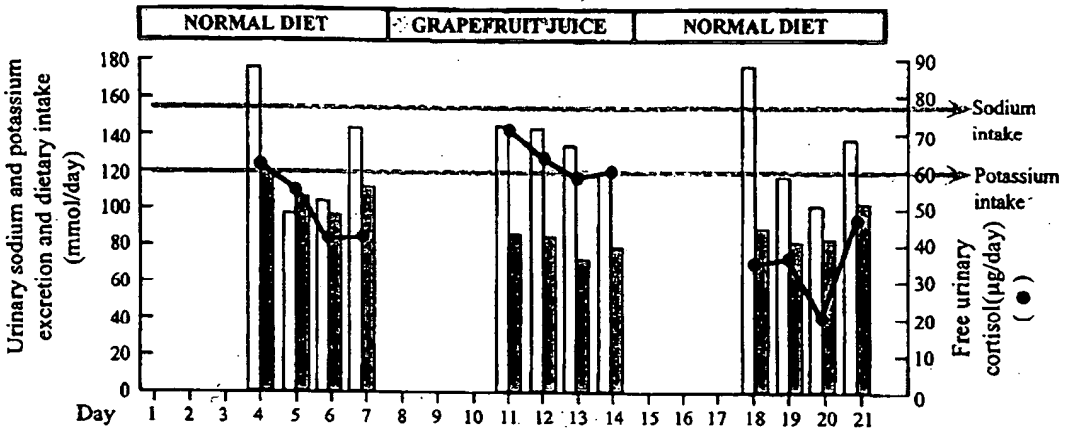
Approval. All human studies were approved by the Cornell Institutional Review Board.

Table I. Inhibition of 11β-OHSD in microsomes of guinea pig kidney by various flavonoids in the presence of NAD or NADP

Flavonoids	*IC_{50} (μmol/L)*	
	NAD	*NADP*
Quercetin*	192 ± 18	355 ± 82
Apigenin*	284 ± 25	125 ± 16
Kaempferol	322 ± 13	293 ± 62
Naringenin*	496 ± 77	264 ± 63
Hesperetin*	769 ± 69	509 ± 45
Naringin*	21,191 ± 4,949	10,550 ± 1,136
Hesperidin	>55,000	>50,000

Data are mean values ± SD.
11β-OHSD, 11β-Hydroxysteroid dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; IC_{50}, drug concentration that inhibited the enzyme by 50%.
*$p < 0.05$; NAD compared with NADP.

SUBJECT A



Urinary cortisone/cortisol	2.7 2.9 3.1 3.1	1.9 2.2 2.2 2.1	3.5 3.0 3.8 2.8	
Plasma cortisol (mcg/dl)	9.5	11.8	12.8	13.5
Plasma renin activity(ng/ml/hr)	0.33	1.4	1.4	1.6

Fig. 4. Values for subject A in metabolic balance study. *Open bars,* Daily urinary sodium excretion; *solid bars,* potassium excretion. The cortisone/cortisol ratios in the grapefruit juice period are significantly different from each normal diet period. The urinary free cortisol during grapefruit juice differs significantly from the first but not the second control period.

RESULTS

In vitro study

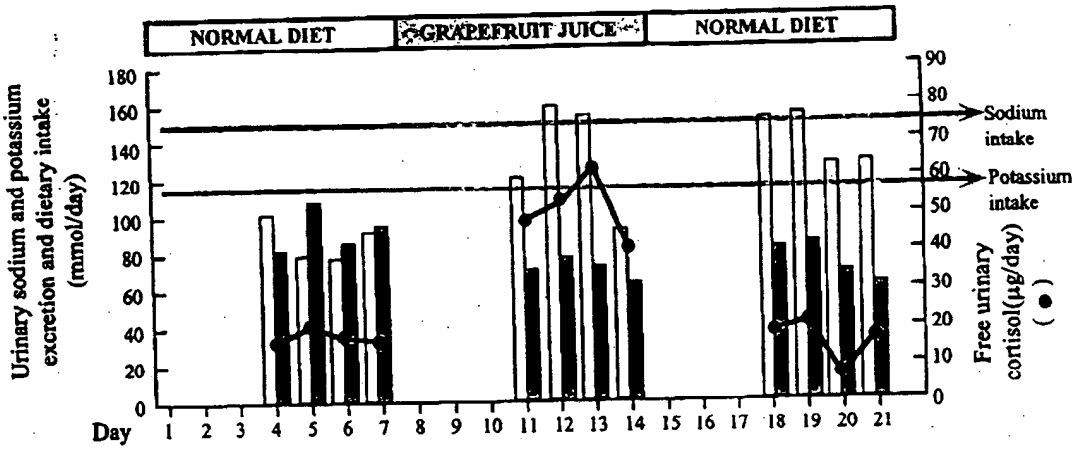
The renal cortex homogenate obtained from male guinea pigs readily converted cortisol to cortisone after 1 hour of incubation at 37° C with NAD or NADP as a cofactor. There was no difference in the conversion rate between NAD and NADP (mean ± SD, 75.1% ± 7.53% with NAD versus 71.0% ± 6.85% with NADP; $p > 0.05$). However, the Michaelis-Menten constant (K_m) values for NAD and NADP calculated from the double reciprocal plots were significantly different (36.4 ± 7.02 μmol/L with NAD versus 57.6 ± 13.1 μmol/L with NADP; $p < 0.05$).

Each flavonoid inhibited the enzyme in a concentration-dependent manner. The inhibition rates for most flavonoids with use of NAD differed from that with use of NADP (Fig. 2). The IC_{50} values of the flavonoids to inhibit the NAD- or NADP-utilizing form of 11β-OHSD are given in Table I. Quercetin was the most potent inhibitor with NAD; apigenin, kaempferol, and naringenin had similar potencies. Apigenin was found to be the most potent inhibitor with NADP, whereas the potency of naringenin, kaempferol, and quercetin were similar. Naringin and hesperidin were poor inhibitors, and their IC_{50} values were much less than that of their aglycons, naringenin and hesperetin. The IC_{50} values of each flavonoid with use of NAD as a cofactor differed from the IC_{50} values with NADP as the cofactor, except for kaempferol.

In vivo dose-response study

The two subjects who drank grapefruit juice showed a dose-dependent decrease in their urinary cortisone/cortisol ratios, indicating inhibition of 11β-OHSD by grapefruit juice (Fig. 3). Each 4-day period was statistically significantly different from the control periods, and the low- and high-dose periods differed in subject 1 statistically and in subject 6 numerically but not statistically.

SUBJECT B



Urinary cortisone/cortisol	3.7 3.8 3.6 2.3	1.8 1.5 1.6 1.8	3.4 3.8 4.3 3.7	
Plasma cortisol (mcg/dl)	7.1	10.9	9.6	10.0
Plasma renin activity(ng/ml/hr)	1.3	3.5	2.5	2.1

Fig. 5. Values for subject B in metabolic balance study. *Open bars,* Daily urinary sodium excretion; *solid bars,* potassium excretion. The cortisone/cortisol ratios and the urinary free cortisol during the grapefruit juice period are significantly different from both control periods.

In vivo metabolic balance study

The actual values for each subject are presented in Figs. 4 and 5. The mean ratios of the urinary cortisone to cortisol fell with grapefruit juice and recovered during the second control period (mean ± SD, 3.27 ± 0.48 during the first control period, 1.88 ± 0.28 during the grapefruit juice period, and 3.52 ± 0.46 during the second control period). Urinary free cortisol levels also were increased during the grapefruit juice period and returned to the control level after subjects ceased to drink grapefruit juice (mean ± SD, 34.3 ± 19.0 for the first control period, 58.2 ± 9.2 for the grapefruit juice period, and 26.3 ± 12.9 for the second control period; $p < 0.05$ for each control period compared with grapefruit juice period). There was a little change in the body weight during the study (67.3 ± 0.4, 67.8 ± 0.3, and 67.5 ± 0.1 kg for subject A and 73.5 ± 0.8, 74.4 ± 0.2, and 74.4 ± 0.1 kg for subject B, in the first control, grapefruit juice, and second control periods, respectively). The urinary sodium and potassium values were variable during the study. There were no significant changes in plasma potassium levels and blood pressure values during the study.

DISCUSSION

Grapefruit juice is known to inhibit the first-pass oxidation of felodipine and nitrendipine,[19,20] presumably because of compounds in the juice that inhibit cytochrome P450 3A. We did this study to learn if it also inhibited another in vivo oxidation, that of 11β-OHSD. We tested several flavonoids present in grapefruit juice for their ability to inhibit 11β-OHSD from guinea pig renal cortex microsomes. The two different isoforms of the enzyme, NAD-dependent and NADP-dependent 11β-OHSD, had different K_m values for cortisol, and the flavonoids had different IC_{50} values for the two forms. We confirmed

the finding of Walker et al.[11] of similar conversion rates for the two forms.

There are a number of flavonoids in grapefruit juice. Naringin is the most abundant flavonoid, present in concentrations of up to 1 mmol/L.[21] It is thought to be converted to the aglycone naringenin in the intestine after oral administration. Because the flavonoids in grapefruit juice inhibited 11β-OHSD in vitro, we evaluated the ability of grapefruit juice to inhibit the enzyme in vivo. Drinking grapefruit juice lowered the urinary cortisone/cortisol ratios in the two investigators and both inpatient subjects, indicating in vivo inhibition of the enzyme. At the doses consumed, it did not change renal electrolyte clearance. Natural licorice in very high doses causes mineralocorticoid effects by inhibition of this enzyme.[4,22,23] We think that grapefruit juice inhibited 11β-OHSD, but the effect was too mild to cause electrolyte changes in these subjects because their urinary free cortisol did not exceed the normal range. A possible alternative explanation is that ring A reduction of cortisol and not 11β-OHSD inhibition is the major cause of the syndrome of apparent mineralocorticoid excess.[23-26]

If the conventional view that 11β-OHSD inhibition is the cause of the syndrome, and if there are differences in different people's enzyme sensitivity to these inhibitors, as we found with different strains of guinea pigs for gossypol inhibition,[8] some people may increase their potassium clearance if they drink large amounts of grapefruit juice. Furthermore, flavonoids are sold in tablet form in health food stores and drug stores. If people take large quantities of flavonoids as dietary supplements, it is possible that the flavonoids may cause sufficient 11β-OHSD inhibition to produce the syndrome of apparent mineralocorticoid excess.

We thank Patricia Danton for her help.

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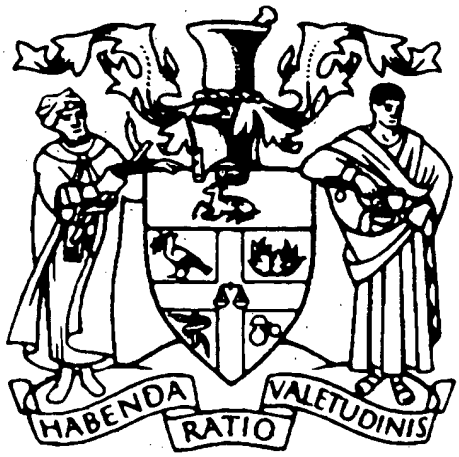

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A Novel 11β-Hydroxysteroid Dehydrogenase Inhibitor Contained in Saiboku-To, a Herbal Remedy for Steroid-dependent Bronchial Asthma

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Abstract—To identify the inhibitor of prednisolone metabolism contained in Saiboku-To, we conducted in-vitro experiments of 11β-hydroxysteroid dehydrogenase (11β-HSD), using rat liver homogenate and cortisol as a typical substrate. We studied the effects of ten herbal constituents on 11β-HSD. Five herbal extracts showed inhibitory activity with *Glycyrrhiza glabra* > *Perillae frutescens* > *Zizyphus vulgaris* > *Magnolia officinalis* > *Scutellaria baicalensis*. This suggests that unknown 11β-HSD inhibitors are contained in four herbs other than *G. glabra* which contains a known inhibitor, glycyrrhizin (and glycyrrhetinic acid). Seven chemical constituents which have been identified as the major urinary products of Saiboku-To in healthy and asthmatic subjects were studied; magnolol derived from *M. officinalis* showed the most potent inhibition of the enzyme (IC50, 1.8×10^{-4} M). Although this activity was less than that of glycyrrhizin, the inhibition mechanism (non-competitive) was different from a known competitive mechanism. These results suggest that magnolol might contribute to the inhibitory effects of Saiboku-To on prednisolone metabolism through inhibition of 11β-HSD.

Saiboku-To is the most popular anti-asthmatic Chinese herbal medicine (Kampo medicine in Japan) and has been used for corticosteroid-dependent asthma to obtain a steroid-sparing effect in prednisolone therapy (Nagano et al 1988). On the basis of animal experiments, the mechanism of action of Saiboku-To has been attributed to hormonal stimulation of the adrenal cortex (Hiai et al 1981; Shimizu et al 1984) and synergistic adjuvant effects on autacoid secretions (Toda et al 1988) or allergic reactions (type I and IV) (Nishiyori et al 1983, 1985).

Recently, we proposed another mechanism which involves suppression of the systemic elimination of prednisolone (Taniguchi et al 1992). This pharmacokinetic effect seemed to result from 11β-hydroxysteroid dehydrogenase (11β-HSD) metabolic enzyme inhibition, because plasma prednisolone/prednisone ratios following Saiboku-To administration increased significantly (Taniguchi et al 1992). Since other Kampo-preparations containing *Glycyrrhiza glabra* did not show an effect on prednisolone pharmacokinetics (unpublished data), the effect of Saiboku-To could not be explained by known enzyme inhibitors such as glycyrrhizin and its aglycone glycyrrhetinic acid, which are contained in *G. glabra*. These observations suggested that Saiboku-To must contain as yet uncharacterized 11β-HSD inhibitors.

In the present study, we carried out in-vitro experiments of 11β-HSD inhibition using cortisol and rat liver homogenate.

Materials and Methods

Materials

Saiboku-To (TJ-96, Tsumura Co., Tokyo, Japan) consists of fine brownish granules containing ten different herbal extracts (Table 1). Original herbs used for the assay were purchased from Uchida Wakanyaku Co. (Tokyo, Japan). The extracts of Saiboku-To and of original herbs were prepared as follows. One gram Saiboku-To or the crushed herb in 15 mL 35% ethanol was gently refluxed for 1 h on a steam bath. After cooling to room temperature, water was added to make a total volume of 10 mL before centrifugation at 1600 *g* for 10 min. The resulting supernatant was used for the assay.

Glycyrrhizin, glycyrrhetinic acid, wogonin, and baicalein were purchased from Wako Pure Chemicals (Osaka, Japan). Magnolol and honokiol were donated by Professor Y. Sashida of Tokyo College of Pharmacy (Fujita et al 1973). Medicarpin and oroxylin A were kindly contributed by Professor T. Nomura of Toho University School of Pharmacy (Tokyo, Japan) and Tsumura Co., respectively. 8,9-Dihydroxydihydromagnolol was prepared by us from magnolol by osmic acid oxidation (Homma et al 1992). Liquiritigenin was isolated from *G. glabra* according to Shibata & Saitoh (1978). Chemical structures of these compounds are given in Fig. 1. Cortisol and cortisone were purchased from Sigma Chemical Co. (St Louis, MO, USA). Other organic and inorganic reagents were of analytical grade.

Rat liver homogenates were prepared in the usual manner: fresh liver was isolated from a male Wistar rat (freely fed, body weight 250 g) and was cut into small pieces. The pieces were homogenized in 10 vol 0·25 M sucrose in a glass homogenizer with a Teflon piston. The homogenates were frozen at −80°C and stored until incubation.

Instruments

Our HPLC system for determination of glucocorticoids in incubation mixtures consisted of a solvent delivery pump (VIP-I, Jasco, Tokyo), a UV-detector (Uvidec-100-III, Jasco), a single pen recorder (Pantos U-228, Nippon Denshi, Tokyo), a sample injector with a loop volume of 100 μL

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Table 1. Herbal composition of Saiboku-To.

Constituent herb	Family	Composition (%, w/w)
Bupleurum falcatum L.	Umbelliferae	20·6
Pinellia ternata Beitenbach	Araceae	14·7
Poria cocos Wolf.	Polyporaceae	14·7
Scutellaria baicalensis Georgi	Labiatae	8·8
Zizyphus vulgaris Lam.	Rhamnaceae	8·8
Panax ginseng C. A. Meyer	Araliaceae	8·8
Magnolia officinalis	Magnoliaceae	8·8
Glycyrrhiza glabra L.	Leguminosae	5·9
Perillae frutescens Britton var. *acuta* Kudo	Labiatae	5·9
Zingiber officinale Roscoe	Zingiberaceae	3·0

(Model 7125, Rheodyne, CA, USA), and a silica gel column (LiChrosorb Si-60, 5 μm, i.d. 4 mm × 250 mm, Merck, Darmstadt, Germany). The mobile phase was a mixture of water/methanol/dichloromethane/n-hexane (0·1/8·0/30·0/61·9 v/v) with a flow rate of 1·5 mL min^{-1}. Detector sensitivity was set at 0·005–0·01 aufs at 245 nm. We used a disposable syringe minicolumn (Extrashot, Kusano Sci. Co., Tokyo) to perform sample injections (Homma et al 1989; Kouno et al 1990).

Determination of 11β-HSD inhibition activity

We measured 11β-HSD activity in rat liver homogenate incubation mixtures, detecting chemical transformation of cortisol to cortisone in the presence of 11β-HSD inhibitors. Oxidation at the C-11 position of the steroid nucleus was kinetically characterized by measuring the conversion rate of cortisol to cortisone in the presence of $NADP^+$ in rat liver homogenate according to the procedure of Monder et al (1989) with minor modification. The incubation mixtures consisted of 620 μL 0·1 M Tris-HCl buffer (pH 8·5) containing 0·014% Triton-X, 50 μL 5 mM $NADP^+$, 100 μL rat liver homogenate, and 200 μL aqueous solution for Saiboku-To and original herbal extracts or 200 μL buffer solution for each chemical such as the known inhibitors (glycyrrhizin and glycyrrhetinic acid) and our candidates isolated from urine of subjects receiving the preparation. These chemicals were dissolved in a buffer solution directly or after pre-solubilization in a small amount of ethanol with a final concentration in incubation mixtures of less than 2%. After 10 min pre-incubation at 37°C, 200 μL 0·3 mM cortisol was added and the resulting mixtures were further incubated for 10 min. The enzyme reaction was terminated by an addition of 100 μL 5% sulphuric acid. Cortisol and cortisone in the mixtures were determined by HPLC using Extrashot as described in our previous papers (Homma et al 1989; Kouno et al 1990). Briefly, 5 μL incubation mixture and 2 μL sodium hydroxide solution were loaded onto Extrashot which was then attached to the sample-loading injector of the HPLC system.

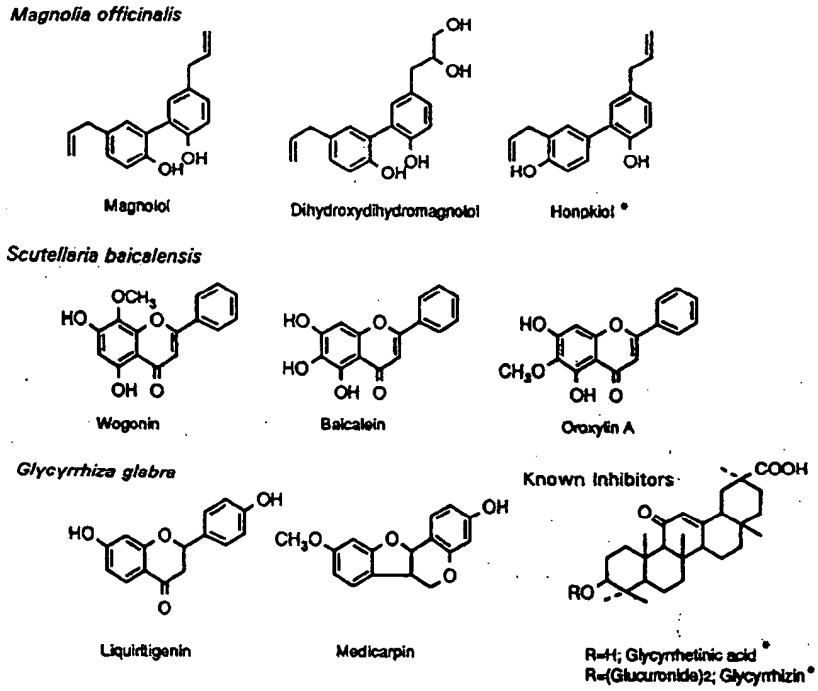

FIG. 1. Chemical structures of test compounds. * These compounds have not been detected in urine following Saiboku-To administration.

Ethanol/dichloromethane (2/98 v/v, 130 μL) was injected into the system through Extrashot using a tuberculin glass syringe. Thus, extraction and injection of the glucocorticoids in the incubation mixtures were achieved simultaneously. The recovery rates of glucocorticoids from the incubation mixture were more than 95% with coefficient of variations less than 5%. Direct peak-height calibration of the test and control mixtures afforded inhibitory activity (% inhibition) of the test materials against 11β-HSD.

Results

Effects of herbal extracts

Effects of original herbal extracts on conversion of cortisol to cortisone by rat liver homogenate were compared with that of Saiboku-To (Table 2). Cortisone production in the reaction mixture was significantly inhibited by Saiboku-To and five original herbal extracts ($P < 0.05$). The magnitude of the inhibition (% inhibition) was in the order Saiboku-To (87.5%) > *G. glabra* (80.8%) > *P. frutescens* (30.9%) > *Z. vulgaris* (27.6%) > *M. officinalis* (19.8%) > *S. baicalensis* (19.1%).

Effects of urinary metabolites of Saiboku-To

Seven candidates (Fig. 1) were tested with respect to the

Table 2. Effects of Saiboku-To and its constituent herbal extracts on 11β-hydroxysteroid dehydrogenase in rat liver homogenate.

	% inhibition[a]	% activity of Saiboku-To
Saiboku-To	87·5±3·4**	100·0
B. falcatum	7·7±5·7	8·8
P. ternata	5·8±4·2	6·6
P. cocos	—	—
S. baicalensis	19·1±11·5*	21·8
Z. vulgaris	27·6±4·0**	31·5
P. ginseng	10·9±6·9	12·5
M. officinalis	19·8±3·7**	22·6
G. glabra	80·8±1·0**	92·3
P. frutescens	30·9±9·6**	35·3
Z. officinale	12·8±8·7	14·6

[a] Data are presented as mean±s.d. of triplicate experiments. *$P < 0.05$, **$P < 0.01$ compared with control.

Table 3. Inhibition of 11β-hydroxysteroid dehydrogenase by urinary metabolites of Saiboku-To and known inhibitors.

	Inhibition (%)	
Inhibitor	10 μM	100 μM
Urinary metabolites of Saiboku-To		
Magnolol	15·1±4·4	43·9±3·0
Dihydroxydihydromagnolol	—	—
Wogonin	—	7·4±0·8
Baicalein	6·8±1·6	14·8±1·6
Oroxylin A	—	5·1±5·5
Liquiritigenin	—	—
Medicarpin	—	12·2±3·3
Known inhibitors		
Glycyrrhizin	81·1±5·4	97·3±1·1
Glycyrrhetinic acid	100·0	

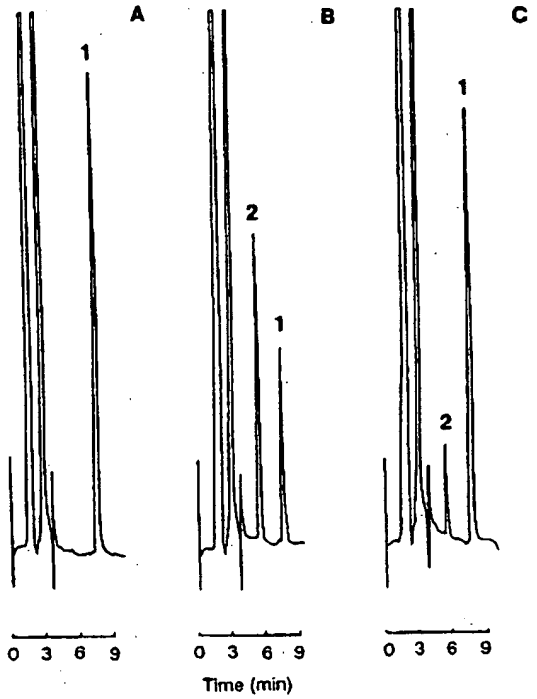

FIG. 2. Chromatographic comparison of the effect of magnolol (100 μM) on transformation of cortisol (peak 1) to cortisone (peak 2) by 11β-hydroxysteroid dehydrogenase. A. Before incubation with magnolol; B. after incubation without magnolol; C. after incubation with magnolol.

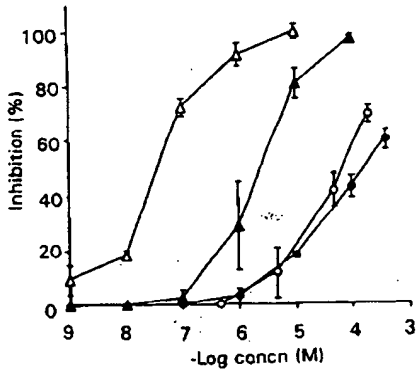

FIG. 3. Dose-dependent inhibitory effects of magnolol (●), honokiol (○), glycyrrhizin (▲), and glycyrrhetinic acid (△) on 11β-hydroxysteroid dehydrogenase. Data are presented as mean±s.d. of triplicate experiments.

effects on rat liver 11β-HSD at concentrations of 10 and 100 μM. The results were compared with those of the known inhibitors, glycyrrhizin and glycyrrhetinic acid (Table 3). Five of seven candidates showed inhibitory activity at 100 μM, although their activities were weaker than those of the known inhibitors. Dihydroxydihydromagnolol in *M. officinalis* and liquiritigenin in *G. glabra* did not show any activity at the test concentrations. Wogonin, baicalein, and oroxylin A (flavonoids derived from *S. baicalensis*), and medicarpin (a

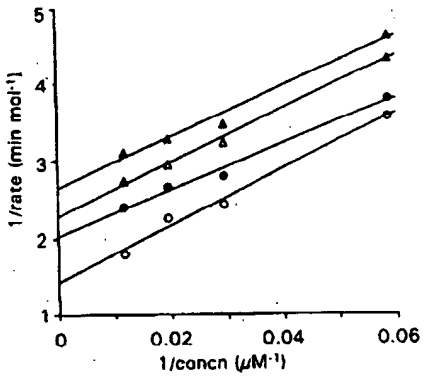

FIG. 4. Lineweaver–Burk double reciprocal plots of initial enzyme velocity and concentration of cortisol in the presence of magnolol at concentrations of 0 (○), 0·1 (●), 0·15 (△), and 0·2 (▲) mM.

homoisoflavonoid in *G. glabra*) showed weak activity. However, considerable inhibition was observed with magnolol, a neolignan derived from *M. officinalis*. A typical chromatogram for determination of the inhibitory activity of magnolol is shown in Fig. 2, where the chemical transformation from cortisol to cortisone was clearly suppressed. The dose-dependent inhibitory effect of magnolol is compared with those of glycyrrhizin and glycyrrhetinic acid in Fig. 3. The IC50 values of magnolol, glycyrrhizin, and glycyrrhetinic acid were $1{\cdot}8\times10^{-4}$, $2{\cdot}6\times10^{-6}$, and $9{\cdot}0\times10^{-5}$ M, respectively. Since *M. officinalis* contains another congener of magnolol, honokiol (not a urinary metabolite), we also examined the effect of honokiol on 11β-HSD and found a dose-dependent inhibitory effect with IC50 of $7{\cdot}0\times10^{-5}$ M (Fig. 3).

Mechanism of magnolol in 11β-HSD inhibition

Fig. 4 shows the inhibitory effects of magnolol on rat liver 11β-HSD. The data were plotted according to the Lineweaver–Burk linear transformation of the Michaelis–Menten equation. The double reciprocal plots on Fig. 4 suggested magnolol has a unique non-competitive inhibitory mechanism. We were unable to estimate an inhibition constant (K_i) of magnolol by the Dixon plot because of this non-competitive inhibition.

Discussion

This paper suggests the presence of several novel inhibitors of 11β-HSD in five constituent herbs. *G. glabra*, *P. frutescens*, *Z. vulgaris*, *M. officinalis* and *S. baicalensis*. Although these inhibitors seem to contribute to in-vitro activity of Saiboku-To, their contributions to prednisolone metabolism during clinical Saiboku-To treatment has been unclear. However, we emphasize the importance of this possibility, since our biologically active compounds in herbal medicine are found in biofluids following administration (Homma et al 1992, 1993a).

In our previous study, we found seven phenolic compounds in urine after oral administration of Saiboku-To (Homma et al 1992, 1993a, b). These compounds seemed to be possible candidates which explain in-vivo effects of Saiboku-To. Five of these compounds showed inhibitory activity against 11β-HSD in-vitro (Table 3). The intensities of those activities were almost equal to those of the corresponding herbal extracts, except that *G. glabra*, containing glycyrrhizin, concealed the effects of liquiritigenin and medicarpin. Magnolol exhibited activity at concentrations higher than 1×10^{-5} M (Fig. 3). Similar activity was also observed in honokiol, a hydroxylated derivative of magnolol isolated from *M. officinalis* but not found as a urinary metabolite of Saiboku-To.

The novel 11β-HSD inhibitors found in this study belong to a class of phenolic compounds, lignans and flavonoids, whose chemical structures are completely different from those of the previously described inhibitors. Unexpectedly, the inhibition mechanism of magnolol seems to be different from those of the known inhibitors, the latter exhibiting competitive inhibition (Monder et al 1989). Although 11β-HSD inhibitors have been considered so far to belong to a limited class of liquorice triterpenoids, the present results suggested that the naturally occurring lignans and flavonoids also possess inhibitory activity through a different mechanism.

Urinary non-conjugated magnolol in responders to Saiboku-To is significantly higher than that in the non-responders (Homma et al 1993a, b). This suggests that magnolol is an important chemical constituent for the clinical effects of Saiboku-To, playing an important role for alteration of prednisolone pharmacokinetics.

The inhibitory effects of liquorice glycosides on 11β-HSD are so marked in animal experiments in-vivo and in-vitro (Monder et al 1989; Mackenzie et al 1990), that Saiboku-To could inhibit 11β-HSD even though the glycyrrhizin content is relatively small. However, the effect of Saiboku-To cannot be explained by glycyrrhizin alone, because another Kampo preparation, Sho-Saiko-To which contains *G. glabra* but not *P. cocos*, *M. officinalis* or *P. frutescens*, did not affect prednisolone pharmacokinetics in healthy subjects (unpublished data). Animal experiments using pure compounds will be needed to clarify the role of lignans and flavonoids on prednisolone metabolism.

Acknowledgements

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